Functional Analysis of Dof Transcription Factors Controlling Heading Date and PPDK Gene Expression in Rice

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Chapter 1

General Introduction

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Introduction

Four millennia ago, people completed the first steps in the domestication of all major cereal crops including rice, barley, wheat and maize (Doebley *et al.* 2006). Rice amongst cereal grasses like maize, millet, barley, sorghum and wheat is the key food for more than half of mankind. The goal of modern rice breeding is to improve cultivars for yield and yield stability under adverse conditions and to improve resistance against biotic stresses. Rice yield is determined by several factors such as the number of tillers per plant, the number of grains per panicle, characteristics of grain filling and grain size. Since the 1960s, the productivity of rice has been largely improved by development of inbred cultivars, which was called the Green Revolution. In the 1970s, Longping Yuan developed the Chinese super-hybrid rice, which increased yield by 30% compared to the parental lines. This development is sometimes called the Second Green Revolution. Heterosis, or hybrid vigor, refers to the improved yield and performance of a hybrid offspring. The molecular genetics and biology behind this biological phenomenon needs to be further investigated in order to reveal the responsible genes.

Rice is an excellent system for plant molecular research as it represents a modest size genome of 430 Mb, containing about 50,000 genes (Goff *et al.* 2002; Tyagi *et al.* 2004). Many rice genomics studies have been initiated in the last decade, some of which took place as international projects like the rice genome research programme (RGP). The rice genome has been completely sequenced (Sasaki *et al.* 2005) for both the japonica (Goff *et al.* 2002) and indica (Yu *et al*. 2002) subtypes and recently low-coverage sequencing of a collection of 3,000 cultivars went public (Li *et al.* 2014). This is excellent material for allele mining and genome wide association studies. Also a large number of T-DNA insertion and transposon-tagged mutants have been produced (Miyao *et al.* 2007), and global gene expression profiles have been obtained using SAGE (Serial Analysis of Gene Expression) or microarray (Bao *et al.* 2005; Li *et al.* 2006). All this will provide useful information and act as a resource platform for further studies on the functional genomics level. However, for the majority of rice genes no functions have yet been demonstrated (Zhang *et al.* 2006). Several genetics studies especially in rice and maize revealed important roles for transcription factors (TFs) and also other regulatory proteins in QTLs determining plant architecture and yield, which were the bases for crop domestication in the past millenia. This chapter will briefly review the recent advances in understanding of the molecular mechanism and roles that TFs have in crop domestication such as apical dominance, seed shattering, tillering, grain size and flowering time.

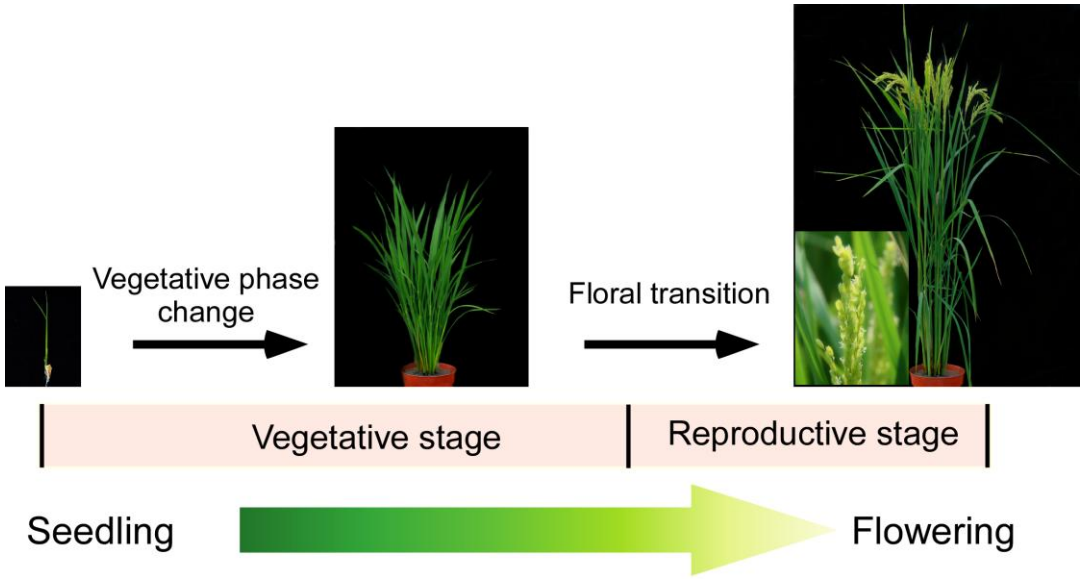

Figure 1. Rice growth and development till flowering.
Flowering is the transition from vegetative stage to reproductive stage, which is important during the rice life cycle. Both environmental signals and endogenous signals affected the transition. Transcription factors play important roles during the life cycle.

Flowering time is the transition from vegetative to reproductive development; it represents one of the major phase changes during the life cycle of a crop such as rice (Figure 1). This transition is initiated by both endogenous and environmental signals. In rice, Heading date genes play roles as endogenous factors controlling the flowering time.

General features of plant transcription factors

Transcription factors (TFs) are proteins or are part of protein complexes, which regulate transcription by binding to specific *cis*-elements in the promoters of target genes and affect the initiation of gene transcription (Ramirez and Basu 2009) and thereby control biological processes. A typical plant TF contains a DNA-binding domain, a transcription-regulation domain and a nuclear localization signal, while some of them also have domains enabling protein-protein interactions (Liu *et al.* 1999). TFs interact with DNA bases in *cis*-acting elements through their DNA binding domain, which in many cases has a basic character because of their amino acid content. Based on the DNA binding domain, the TFs can be divided into different families. For example, there are 57 TF families identified in sorghum and 58 in maize (Perez-Rodriguez *et al.* 2010) and 58 in Arabidopsis (Jin *et al*. 2014). TFs not only can bind to DNA but also regulate gene expression on the transcriptional level. Some TFs have a transcription repression domain by which they can repress target gene expression, whereas others have a transcription

activation domain by which they can activate target gene expression. Another aspect of TF functionality is their intracellular localization. Translation of a TF-coding mRNA occurs in the cytoplasm, but transcription occurs in the nucleus, so the TFs have to enter the nucleus in order to be able to regulate the transcription of target genes. For entering the nucleus, TFs usually contain an NLS (Nuclear Localization Signal) which is characterized by a core peptide enriched in arginine and lysine (Boulikas 1994).

Many plant TFs form heterodimers or homodimers and as a consequence of the dimer interaction show more specificity in DNA recognition and functionality (Lu *et al.* 2002). Some TFs have oligomerization domains which are normally adjacent to or overlap with the DNA binding domain (Katagiri *et al.* 1992). The oligomerization domains and DNA binding domains display a tertiary structure which determines critical aspects of TF activity (Guiltinan and Miller 1994). The variations in oligomerization increase the versatility of the transcription machinery and of modulation of gene expression. Hence, studies of plant TFs are important not only for expanding our knowledge of plants but also for future challenges of generating genetically modified crops with superior characters.

Transcription factors play important roles in crop domestication

Over the past decade, several TFs have been identified, which control some of the important morphological changes associated with crop domestication. For instance, one of the biggest differences between maize and its progenitor, teosinte, is the difference in apical dominance (Doebley *et al.* 1997; Doebley 2004). Modern maize cultivars have a single stalk and one ear on the top of the plant, but wild type non-domesticated teosinte has multiple stalks and long branches. TEOSINTE BRANCHED1 (Tb1), a member of the TCP TF family, largely controls these differences between maize and teosinte. Tb1 can repress the outgrowth of the axillary meristems and branch elongation via a negative effect on the cell cycle (Dorweiler and Doebley 1997; Clark 2005; Camus-Kulandaivelu *et al.* 2008; Tsiantis 2011). Another well-described example of the role of a TF in crop domestication is Tga1. This is a member of the squamosa-promoter binding protein (SBP) TF family and was identified as a TF that played an important role in maize domestication (Wang *et al.* 2005). Tga1 might sit at the top of a cascade of transcription regulators, because some of the members of this family can regulate the expression of *MADS* TFs (Cardon *et al.* 1999). As major regulator of other TFs, the *Tga1* gene affects diverse traits such as cell lignification, three dimensional organ growth, and organ size and silica deposition (Dorweiler and Doebley 1997). Other *SBP* genes with important roles in QTLs for yield components in rice are *OsSPL14* and *OsSPL16* which respectively control grain number and tiller formation

(Miura *et al.* 2010; Liang *et al.* 2014; Wang *et al.* 2012). Likely these genes were also important for crop domestication.

Another important trait in cereal domestication is shattering of seeds. Normally seeds shatter easily from the rice panicle or the wheat spike but for agricultural purposes it is required that they stay attached during harvest. A number of QTLs controlling this process have been identified. *Sh4* and *qSH1* are two key genes required for reduced seed shattering during rice domestication. *Sh4* encodes a protein with homology to Myb3, which is a TF from the MYB domain family and controls whether the seed fall off the plant as in wild rice or adhere to the plant like in cultivated types. Transgenic experiments showed that a single amino acid change converts rice from shattering to non-shattering types. The decrease in expression of the cultivated allele compared to the wild type allele is also important in the domestication (Li *et al.* 2006). *qSH1* is a BEL1-type homeobox gene (Zhou *et al.* 2012). The single nucleotide change in cultivated rice interferes with a *cis*-regulatory element in the promoter required for the expression of *qSH1* in the abscission layer at the base of the rice grain, which is needed for the grain to break away from the axis of the panicle (Konishi *et al.* 2006; Zhou *et al.* 2012). In wheat, *Q* affects a range of traits including the tendency of the spike to shatter, the tenacity of the chaff surrounding the grain, and whether the spike is elongated as in wild wheat or compact like the cultivated forms after wheat domestication (Simons *et al.* 2006)

Tillering is also an important agronomic trait for determining rice yield, because it greatly affects the panicle number which is a key component of rice production and is one of three main yield components (Sakamoto and Matsuoka 2008; Xing and Zhang 2010). *MOC1* (*MONOCULM1*) was identified as important gene in the control of rice tillering. The *moc1* mutant has only one main culm without any other tillers because of a defect in the formation of tiller buds. *MOC1* does not code for a TF but encodes a putative GRAS family protein and is expressed mainly in the axillary buds and functions to initiate axillary buds and to promote their outgrowth (Li *et al.* 2003). *DLT* (*DWARF AND LOW-TILLERING*) also encodes a GRAS family protein and the knockdown mutant showed a reduction in tiller numbers associated with altered expression of several brassinosteroid signalling genes (Li *et al.* 2010; Tong *et al.* 2012). Recently, it was found that OsMADS57 interacts with OsTB1 (TEOSINTE BRANCHED1) and targets D14 (Dwarf14) to control tillering (Guo *et al.* 2013).

In addition, grain size is important for rice yield potential, and grain size has been an important target in rice breeding (Takeda and Matsuoka 2008). Gene *OsSPL1*6 controls grain size and shape in rice, which can improve grain quantity and yield (Wang *et al.* 2012). The over-expression of *PGL1* (*POSITIVE REGULATOR OF GRAIN LENGTH*), which is a TF of the bHLH

family, increases grain length and weight due to increased cell length of lemma and palea. Moreover, *APG* (*ANTAGONIST OF PGL*) encodes a typical DNA-binding basic helix-loop-helix protein, which acts as an interaction partner of PGL1. Knock down of *APG* led to increased grain length, which suggested that APG was a negative regulator of grain length and weight, and PGL1 was an inhibitor of APG (Heang and Sassa 2012). Other genes involved in determining grain size are *GS3* (Mao *et al.* 2010), *OsPPKL1* (Zhang *et al.* 2012) and *GS6* (Sun et al. 2013).

Another very important trait in rice domestication is the diversification of flowering time (FT). Rice is a typical short day (SD) plant and it needs a suitable photoperiod for flowering in the correct time which is important to get seeds. In rice, OsGI-Hd1-Hd3a is the major pathway controlling flowering time (Yano *et al.* 2000; Hayama *et al.* 2002; Kojima *et al.* 2002). The genes orthologous to *CO* and *FT* in rice were identified as flowering-time QTLs and named *Hd1* and *Hd3a* accordingly. Under the SD condition, *Hd1* has functions downstream of *Hd3a* (Kojima *et al.* 2002; Takahashi and Shimamoto 2011), encoding a mobile flowering signal and promotes floral transition (Tamaki *et al.* 2007). *RFT1*, a gene homologous to *Hd3a*, is also essential for flowering in rice and it promotes flowering in the absence of Hd3a (Komiya *et al.* 2008). Both *RFT1* and *Hd3a* are floral activators under SD condition and they act up-steam of *OsMADS14* and *OsMADS15* (Komiya *et al.* 2008). *Early heading date 1* (*Ehd1*), a B-type regulator, promotes rice flowering under SD condition. No ortholog of *Ehd1* was found in the *Arabidopsis* genome (Doi *et al.* 2004). *Ehd2*, encoding a putative TF with zinc finger motifs, acts as a flowering promoter by up-regulating *Ehd1* under SD condition (Matsubara *et al.* 2008). *OsMADS51* is also a short day flowering promoter which functions upstream of *Ehd1*, *OsMADS14* and *Hd3a* (Kim *et al.* 2007). *OsMADS50*, which also belongs to the *MADS* TF family, also acts as an important flowering activator upstream of *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18* and *Hd3a* (Lee *et al.* 2004). Yeast two hybrid screening and pull down experiments showed that one of the MADS-box genes controlling flowering development in rice, OsMADS1, interacted with OsMADS14 and OsMADS15 together (Lim *et al.* 2000).

Data from the Arabidopsis genome project indicated that more than 5% of the genes encode TFs (Riechmann *et al.* 2000), and in rice about 4% of the genes encode TFs (Tyagi *et al.* 2004). Since the number of non-TF genes is 20-25 larger than the number of TFs, the conclusion is that a single TF is able to regulate the expression of large sets of other genes. The utilization of TFs provides advantages in genetic engineering in that a set of genes can be manipulated at the same time. Plant TFs might act as a hub in the complicated net of gene expression and regulation. Research on TFs can reveal the molecular mechanism of gene expression and regulation, and the

biological processes which are controlled by it. Furthermore, the study of transcription factors might lead us to meet future challenges in crop improvement.

In rice, in total 58 TF families are corresponding to 1,981 and 2,408 putative transcription factors in the *indica* and *japonica* subspecies respectively (Jin *et al.* 2014). One of these, the so-called Dof (DNA-binding with one finger), family is a plant specific group of zinc-finger TFs (Yanagisawa 1995). Although there are several reports about functions of Dof TFs in plants, the functions of most rice Dof genes is still far from clear, and they need to be further investigated and explored for their potential in plant breeding. The characteristics of this gene family and known functions are discussed in the next section of this chapter.

Dof transcription factor genes

The first protein identified with a Dof domain was MNB1a in maize which is identical to Dof1 and which can interact with the Cauliflower Mosaic Virus (CaMV) 35S promoter (Yanagisawa and Izui 1993; Yanagisawa 1995). Dof genes seem to be implicated in many different biological processes (Yanagisawa 1995, 1996, 1998). Dof domain proteins are TFs with a highly conserved DNA-binding domain, which presumably includes a single C_2-C_2 zinc finger (Figure 2). They form an important plant-specific transcription factor family (Yanagisawa 1998, 2002, 2004). In *Arabidopsis,* there are 36 annotated TFs belonging to the Dof family, whereas there are 30 Dof proteins in the rice genome (Lijavetzky *et al.* 2003; Noguero *et al*. 2013). Although mainly found in higher plants, Dof proteins have also been discovered in the unicellular alga *Chlamydomonas reinhardti,*mosses and gymnosperms. This indicates an ancient origin and the possibility of diversification throughout plant evolution. As we know, homologous sequences are paralogous if they were created by genome duplication, and they are orthologous if they are copies of a single gene in different species. Paralogs usually display different functions, while orthologs largely retain the same function (Tatusov *et al.* 1997). There is very little literature about rice Dof TFs, but a lot of data are available about Dof TFs in other species such as Arabidopsis and maize. Theinformation about the Dof transcription factors from these other species will be helpful to assay the homologous Dof gene in rice.

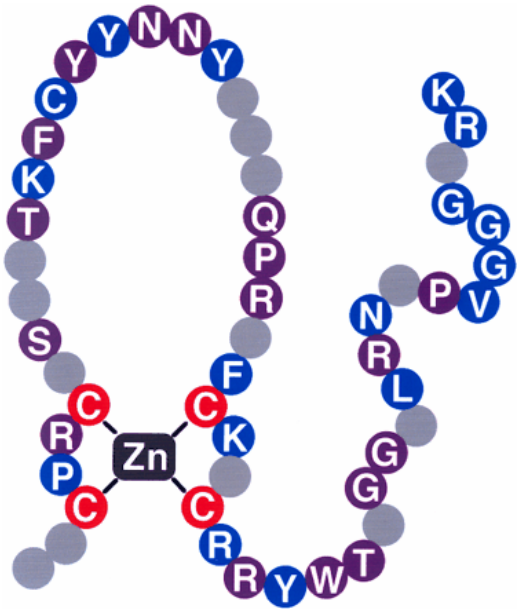

Figure 2. A proposal for the structure of the Dof domain (Yanagisawa, 2004). Absolutely conserved cysteine residues that presumably coordinate zinc and other amino acid residues conserved among all known Dof domain proteins are shown in red and purple, respectively. Well-conserved residues are shown in blue, and redundant residues are shown in gray.

The highly conserved Dof zinc finger domain, normally locates at the N-terminus, and the amino acid sequences outside the Dof domain are very diverse and show little homology (Yanagisawa 2002). The specific DNA sequences to which the Dof proteins bind have been verified by *in vitro* and *in vivo* experiments. All Dof proteins analyzed so far recognize an AAAG motif or its revise complementary sequence, CTTT, in the *in vitro* DNA binding assays (Yanagisawa 2002). The only exception is the pumpkin protein AOBP (Kisu *et al.* 1998), which takes AGTA repeats as DNA-binding motif. The Dof domain alone is able to confer the sequence specific DNA interaction (Yanagisawa 1995; Kisu *et al.* 1998), so the regions outside the Dof domain do not play a major role in the interaction with DNA. It has been reported that maize Dof1 can bind an AAAG motif in the nucleosome reconstructed *in vitro* as well as the motif on naked DNA, although the binding was dependent on the position of the AAAG motif in nucleosome (Cavalar *et al.* 2003). Moreover, the specific interaction of Dof proteins with the AAAG motif has been also verified *in vivo*. Dof proteins can bind and activate transcription from a synthetic promoter containing an AAAG motif or certain native promoters embracing an AAAG motif in protoplasts isolated from plants (Yanagisawa and Sheen 1998; Yanagisawa 2000). Furthermore, mutational assays showed the physiological significance of the AAAG sequences in the putative target promoters, such as the promoter of the maize *PEPC* gene and the maize *PPDK* gene

(Yanagisawa 2000). All Dof domains have four conserved cysteine residues, and metal chelators inhibit DNA-binding of maize Dof1 and some other Dof proteins *in vitro* (Yanagisawa 1995, 2002). Replacement of the conserved cysteine residues with alanine or serine completely abolished DNA-binding of the Dof proteins *in vitro* (Yanagisawa 1995) and *in vivo* (Mena *et al.* 1998). Although there are still no structural biological data, these results strongly indicate that the Dof domain is essential for DNA-binding.

The Dof domain was originally identified as a DNA-binding domain, but now it is regarded as a bifunctional domain for both DNA-binding and protein-protein interactions with other classes of transcription factors (basic domain-leucine zipper proteins, bZIP proteins) and non-histone nuclear proteins called high-mobility group (HMG) proteins. The first protein-protein interaction was found with an Arabidopsis Dof domain protein (OBP1), which was found interacting with bZIP proteins associated with stress responses (Zhang *et al.* 1995; Chen *et al.* 1996). Another example of a Dof-bZIP interaction was shown for a maize Dof domain protein (PBF) and a maize bZIP protein, Opaque2 (O2) which regulates endosperm-specific gene expression (Vicente-Carbajosa *et al.* 1997). The Dof domain also interacts with the conserved DNA-binding domain (the HMG-box) of HMG proteins, abundant non-histone nuclear proteins likely functioning in transcription and DNA replication. The binding of maize Dof1 and Dof2 to DNA was promoted by HMG protein (Yanagisawa 1997). Although protein-protein interactions mediated by the Dof domain were shown with only a few Dof proteins till now, all Dof domains might confer protein-protein interactions because the Dof domain is highly conserved.

As the amino acid sequences outside the Dof domain are divergent, the Dof domain proteins also play diverse roles in plants. These roles include responses to plant hormones such as gibberellin (Washio 2001; Mena *et al.* 2002) and auxin (DePaolis *et al.* 1996; Kisu *et al.* 1998), stress responses (Corrales *et al*. 2014), tissue specific expression (Yanagisawa 1998; Plesch *et al.* 2001) and photosynthesis (Yanagisawa and Sheen 1998; Yanagisawa 2000).

Maize Dof1 was associated with expression of multiple genes involved in carbon metabolism (Yanagisawa 2000). It can recognize the AAAG motif in the maize *C4PPDK* promoter and activates the expression of this gene (Yanagisawa 2000), whereas Dof2 acts as a tissue-specific repressor (Yanagisawa and Sheen 1998). Further *in vitro* experiments showed that the chromosomal high-mobility group (HMG) protein HMG5 can facilitate nucleosome binding of the transcription factor independent from the position of the recognition sites (Cavalar *et al.* 2003). Nowadays, *Dof* genes and functions have been described in many other plant species. Two soybean *Dof* genes, *GmDof4* and *GmDof11* can enhance the lipid contents in transgenic

Arabidopsis by up-regulating genes which are associated with the biosynthesis of fatty acids (Wang *et al.* 2007). *SRF1*, a Dof gene from sweet potato, modulates the carbohydrate metabolism in the storage roots by negative regulation of *Ibbfruct2*, a vacuolar invertase gene (Tanaka *et al.* 2009). In *Triticum aestivum* (wheat), members of the Dof transcription factor family are associated with light-mediated gene regulation, including involvement in photosynthesis (Shaw *et al.* 2009), and TaDof1 might be related to the up-regulation of C4 pathway-related genes functioning in heterosis (Chen *et al.* 2005). In Arabidopsis, *Dof* genes play important roles in different biological courses, for instance *OBP1* in the cell cycle regulation (Skirycz *et al.* 2008) and *COG1* and *OBP3* in regulating of phytochrome signaling (Ward *et al.* 2005), the *CDF1*, *CDF2* and *CDF3* genes in photoperiodic control of flowering (Fornara *et al.* 2009), the *DAG1* and *DAG2* genes in seed germination (Gualberti *et al.* 2002).

The Dof TFs in the rice genome (Lijavetzky *et al.* 2003; Noguero *et al.* 2013) have been classified into four major clusters, but thus far only OsDof3 and OsDof12 have been well described by functional analysis (Washio 2001; Engelmann *et al.* 2003; Li *et al.* 2009). OsDof3 plays an important role in the gibberellin-mediated expression of genes involved in seed germination (Washio 2001; Engelmann *et al.* 2003), is expressed in maturing endosperm and coordinately expressed with seed storage protein genes. OsDof3 was also found involved in the quantitative regulation of genes which are expressed in the endosperm in cooperation with this *RISBZ1* gene (Yamamoto *et al.* 2006). OsDof12 is a promoter of the rice heading date by up-regulating the genes *Hd3a* and *OsMADS14* under Long Day (LD) condition (Li *et al.* 2008; 2009). OsDof9 and OsDof18 were reported to be expressed in rice aleurone layer of grains (Washio 2001). Except for the few reported Dof genes, most of the Dof transcription factor genes have not been investigated in rice till now. Functions of most of the rice Dof transcription factors need to be researched and unraveled. In the thesis we studied two of the rice Dof genes, *OsDof24* and *OsDof25*, which are related to *OsC4PPDK* transcription and regulation, heading date and seeds storage proteins in rice.

Outline of this thesis

The aim of this thesis was to make a start with studying the molecular basis for heterosis in Chinese Super hybrid rice by focusing on the transcriptional regulation of *C4PPDK* which is an important gene in photosynthesis. Heterosis, hybrid vigor, or outbreeding enhancement, is the improved or increased function of any biological quality in a hybrid offspring. Chinese Super hybrid rice shows typical heterosis than the parental lines, which offer a good system for investigation of heterosis. Previous studies showed that several genes involved in C4 photosynthesis including *C4PPDK*, were up-

regulated in hybrid rice (Bao *et al.* 2005) compared to the parental lines. Based on the hypothesis that certain transcription factors may be responsible for these expression polymorphisms, we developed experiments where we finally identified two Dof proteins as regulatory factors of rice C4PPDK.

Chapter 1 presents a general introduction to the factors involved in crop domestication and the specific role of transcription factors in this process. **Chapter 2** describes the functional analysis of a Dof-type transcription factor, named *OsDof24,* from rice. Over-expressing *OsDof24* in rice resulted in plants with a reduced plant height and delayed flowering time, but down-regulation using an RNAi approach did not render any obvious phenotype. Detailed transcriptional profiling of heading date-related genes in *OsDof24*-overexpressing transgenic rice showed that OsDof24 can function in the delay of the heading date by down-regulating transcription of genes *Hd3a* and *RFT1*, which are two critical genes controlling this process. **Chapter 3** describes the role of *OsDof25* in the regulation of *OsC4PPDK.* Interactions between *OsDof25* and *OsC4PPDK* were confirmed in experiments involving EMSA, yeast one-hybrid analysis and transient expression in rice protoplasts. The regulatory role of *OsDof25* in *OsC4PPDK* expression was confirmed using *OsDof25* misexpression plants. The results suggested that OsDof25 is a trans-activator of the *OsC4PPDK* gene in rice. **Chapter 4** describes the effects of OsDof24 and OsDof25 on the content of rice seeds storage proteins. First the interaction between both OsDof24 and OsDof25 with the *GluB-1* promoter was investigated by a set of *in vitro* and *in vivo* experiments. Results showed that both OsDof24 and OsDof25 can affect seed storage protein content probably by activating the transcription of the *GluB-1* promoter. Finally, in **Chapter 5**, a general discussion of the results from Chapters 2, 3 and 4 is presented.

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Chapter 2

Functions of OsDof24 in determining flowering time

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Key words (listed in order of importance): Dof, transcription factor, heading date, rice

Abstract

OsDof24 belongs to the so-called Dof (DNA-binding with one finger) transcription factor family which is involved in a variety of biological processes in plants. Transgenic rice overexpressing *OsDof24* showed a delay of flowering time under photoperiod of 12h light/12h. RT-PCR and qPCR profiling on these plants showed that the flowering time genes *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1* were down-regulated, whereas *OsMADS50* was up-regulated, and the expression level of *Hd1*, *OsMADS15*, *OsMADS18*, *OsMADS51* and *Ehd1* were not changed. Bioinformatics analyses of the *OsMADS1*, *OsMADS14*, *Hd3a*, *RFT1* and *OsMADS50* promoters confirmed the presence of Dof binding sites. Based on these results, we hypothesize that *OsDof24* may be involved in determining flowering time by down-regulating the expression of several flowering time genes, especially *Hd3a* and *RFT1* which are two key genes in the flowering time pathway in rice.

Introduction

One of the most important traits in rice cultivation is flowering time because it determines if the crop fits in the local season. The exact moment of flowering time is determined by the genetic background and the interactions with environmental factors (Searle and Coupland 2004; Baurle and Dean 2006; Imaizumi and Kay 2006; Yu *et al.* 2011). One of the most important environmental factors that affect flowering time, apart from temperature, is the change in day length (photoperiod) (Imaizumi and Kay 2006). According to photoperiodic responses, plants are grouped into three classes: long-day (LD) plants, in which flowering occurs when the day becomes longer than a crucial length; short-day (SD) plants, in which flowering occurs when the day becomes shorter; and day-neutral plants in which flowering is not regulated at all by day length (Salisbury 1985). Normally if the light is more than 14 hours during a day, it is considered a long day (LD) condition, and if the dark is more than 14 hours, it is considered a short day (SD) condition. There are different factors influencing the photoperiodic pathway such as photoreceptors, the circadian clock and floral integrator genes (Simpson and Dean 2002; Hayama *et al.* 2003; Imaizumi and Kay 2006). In Arabidopsis, the *GI-CO-FT* related pathway is a critical mechanism in the photoperiod pathway (Putterill *et al.* 1995; Valverde *et al.* 2004). *GIGANTEA* (*GI*) integrates cellular signals from light sensory transduction and circadian clock and then activates *CONSTANS* (*CO*). Under LD conditions, *CO* promotes flowering by regulating transcription of *FLOWERING LOCUS T* (*FT*) which encodes a mobile protein called florigen which promotes flowering under both SD and LD conditions (Samach *et al.* 2000; Tiwari *et al.* 2010; Kardailsky *et al.* 1999). *CYCLING DOF FACTOR 1* (*CDF1*), a Dof transcription factor, is expressed in the same tissues with *CO* and it binds to the *CO* promoter thereby acting as repressor of *CO* transcription. In turn FLAVIN-BINDING, KELCH REPEAT,F-BOX1(FKF1) controls the expression of *CO* by mediating the degradation of CDF1 (Imaizumi *et al.* 2005).

The GI-CO-FT regulatory module controlling flowering time is conserved as OsGI-Hd1-Hd3a (Yano *et al.* 2000; Hayama *et al.* 2002; Kojima *et al.* 2002) in rice. *OsGI*, the ortholog of Arabidopsis *GI* regulates expression of *Hd1* (*Heading date 1*) and *OsMADS51* (Hayama *et al.* 2002, 2003; Kim *et al.* 2007). Under SD conditions, Hd1 promotes flowering by up-regulating expression of *Heading date 3a* (*Hd3a*), but under LD conditions it suppresses the expression of *Hd3a* thereby delaying flowering time (Kojima *et al.* 2002; Hayama *et al.* 2003). *Hd3a* in fact encodes for florigen, the long searched for mobile flowering signal, and promotes floral transition by regulating the expression of its downstream genes *OsMADS14* and *OsMADS15* (Kojima *et al.* 2002; Tamaki *et al.* 2007). *RICE FLOWERING LOCUS T1 (RFT1*), a gene homologous to *Hd3a*, also encodes a mobile flowering signal and promotes floral transition under SD condition. The expression of *RFT1* is normally very low plants but increased in *Hd3a* RNAi plants. Both *Hd3a* and *RFT1* are essential for flowering in rice (Komiya *et al.* 2008). *Early heading date 1* (*Ehd1*) encoding a B-type regulator promotes rice flowering under SD condition (Doi *et al.* 2004). There is no orthologous gene in the *Arabidopsis* genome of *Ehd1*, so there are differences in the pathway controlling flowering time between these species. In this pathway, *Ehd2*, a putative transcription factor with zinc finger motifs, acts as a flowering promoter by up-regulating *Ehd1* under SD condition (Matsubara *et al.* 2008). *OsMADS51* is a short day flowering promoter functioning upstream of *Ehd1*, *OsMADS14* and *Hd3a* (Kim *et al.* 2007). *OsMADS50* acts as an important flowering activator under LD condition of the heading date related genes *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18* and *Hd3a* (Lee *et al.* 2004; Komiya *et al.* 2009). Recently, it was reported that *OsDof12* regulates flowering time by regulating expression of *Hd3a* and *OsMADS14* (Li *et al.* 2009). In tomato, Dof transcription factors (SlCDFs) were implicated in abiotic stress tolerance and flowering time (Corrales *et al.* 2014). Taken together, it is clear that flowering time is a complex process determined by many different genes with activating or repressing functions.

Dof proteins are members of a plant-specific transcription factor family (Yanagisawa 2004), which share a highly conserved DNA-binding domain (Yanagisawa 2000). The name of Dof is coined from DNA-binding with one C2-C2 zinc finger (Umemura *et al.* 2004). There are two major domains in the Dof transcription factors: an N-terminal conserved DNA-binding domain and a C-terminal domain for transcriptional regulation (Kang and Singh 2000; Yanagisawa 2000). The Dof domain is known to be a bi-functional domain which participates in not only DNA-binding but also in protein-protein interactions (Yanagisawa 1997). The highly conserved Dof domains in different Dof proteins suggested that Dof proteins display similar DNA-binding specificity. In fact, an AAAG motif or its reversibly orientated sequence, CTTT, are always the targets for Dof proteins, except for a pumpkin Dof protein (AOBP) which recognizes an AGTA repeat as its binding motif (Yanagisawa 2000). The highly variable amino acid sequences outside the Dof domain suggested that the functions of Dof proteins might be diverse. Since the first Dof protein, maize

Dof1, was identified in maize (Yanagisawa 2000), numerous Dof proteins have been reported playing important roles in diverse plant-specific processes such as response to plant hormones including gibberellin (Mena *et al.* 2002), auxin (Kisu *et al.* 1997, 1998; Baumann *et al.* 1999) and salicylic acid (Kang and Singh 2000; Kang *et al.* 2003); plant development including seed germination (Dong *et al.* 2007; Moreno-Risueno *et al.* 2007); stomata guard cell opening (Plesch *et al.* 2001); light responses (Yanagisawa 2000) and grain size control (Masao *et al*. 2009).

In the rice genome 30 putative Dof transcription factors have been identified (Lijavetzky *et al.* 2003), but so far only *OsDof3* and *OsDof12* were studied in detail (Yano *et al.* 2000; Li *et al.* 2009). In this study, we identified *OsDof24*, a Dof transcription factor, and investigated its functions in determining the heading date. *OsDof24* was assigned to the group C-type Dof transcription factors, which are related to seed maturation, seed germination and vascular development (Shigyo *et al.* 2007). In this chapter, we demonstrate that *OsDof24* is a key regulator of flowering time by down-regulating the flowering time genes *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1*.

Results

OsDof24 belongs to the rice Dof transcription factor family

OsDof24 is one of the 30 genes encoding the Dof transcription factor family in rice (Lijavetzky *et al.* 2003; Riano-Pachon *et al.* 2007). The gene encoding OsDof24 obtained from hybrid rice combination Liangyou 2186 is 831 bp long which is the same as a TIGR prediction (LOC_Os08g38220 in MSU osa1 release 7) and encodes for a 276 amino acid protein. There is no intron in the *OsDof24* genomic sequence. Figure S1 shows the sequence and structure of OsDof24 including the Dof domain and the four cysteine residues characteristic for the Dof zinc finger.

To confirm that OsDof24 is a nuclear-localized transcription factor, a GFP-tagged fusion protein under control of the CaMV 35S promoter was made, with the GFP ORF fused at the C-terminus of the full-length OsDof24 protein (construct 35S::GFP-OsDof24). This construct was introduced into rice protoplasts by PEG-mediated transient transformation and analyzed using CSLM, where a 35S::GFP construct was used as control. As shown in Figure 1A, a GFP signal was detected specifically in the nucleus of 35S::OsDof24-GFP transformed cells, whereas in the control, the GFP signal was located primarily in the cytoplasm. These data support that OsDof24 is a nuclear-localized protein.

Expression profile of *OsDof24*

The expression profile of *OsDof24* was analyzed using qPCR in wild-type rice and by GUS staining in transgenic plants equipped with a promoter-GUS reporter construct. The expression level of *OsDof24* was found highest in two week old seedlings and

lowest in stems of mature plants, respectively (Figure 1B) Expression at different levels was seen in penultimate leaves, leaf sheath, stems, roots and panicles.
Using the *OsDof24* promoter-*GUS* construct, GUS activity was detected in embryogenic calli, flag leaves, panicles, shoot apical meristem (SAM). GUS activity was also visible in developing seeds and peaked at 10 DAF (days after fertilization). There are also GUS signals in stem, roots and two days old germinating seeds. Cross-sections of leaf tissue and the SAM showed that the GUS signal was highest in cells associated with the vascular bundles (Figure 1C). In panicles, *OsDof24* was expressed in the palea and lemma, which enclose the other flower organs and the developing grain (Figure 1C). The GUS activity in ProOsDof24::GUS transgenic rice is in accordance with the expression profile of *OsDof24* as analyzed using qPCR.

Delay of flowering time in *OsDof24* overexpressing transgenic rice

To further elucidate the function of *OsDof24*, transgenic overexpression plants were made. For this, a binary vector, *ProGOS2::OsDof24*, was constructed by expressing the *OsDof24* under control of the constitutive *GOS2* promoter (De Pater *et al.* 1992; Ouwerkerk *et al.* 2001). Before phenotyping, single copy plants originating from three independent callus lines were genotyped using qPCR. As shown in Figure 2c, the qPCR analysis of the T_1 plants showed that upregulation of expression of *OsDof24* ranged from 3 to 10 times. Flowering time was recorded as the first day the first flower appeared compared to the day the plants were seeded. Plants from all three lines were phenotyped in the greenhouse, and showed two weeks later flowering than the controls, which were non-transgenic azygous plants segregated from the T_0 generation (Figure 2a). Statistical analysis showed that the *OsDof24-OX* plants were flowering at least two weeks later compared to the controls. For instance, line OX1 was flowering 38 days later than the control and lines OX2 and OX3 were flowering respectively 15 and 20 days later. Furthermore, another obvious observation was that the leaves of the *OsDof24-OX* plants were growing only in one plane whereas normally wild type rice leaves grow in all directions (Figure 2b). Obviously, *OsDof24-OX* T_1 plants were smaller than the controls, (Figure 2a). The average height of the *OsDof24-OX* plants was 14 cm to 44 cm shorter (26 cm in average) than the control. The internode length was 8 cm to 17 cm shorter but the leaf number per tiller was at least three more than the control (Figure 2, Table 1). Thus although the plants had more leaves and thus (inter)nodes, they were still shorter because internode length was reduced. These results indicated that *OsDof24* does not only have a role in determining flowering time but also in other major traits such as plant height, leaf number and internode length.

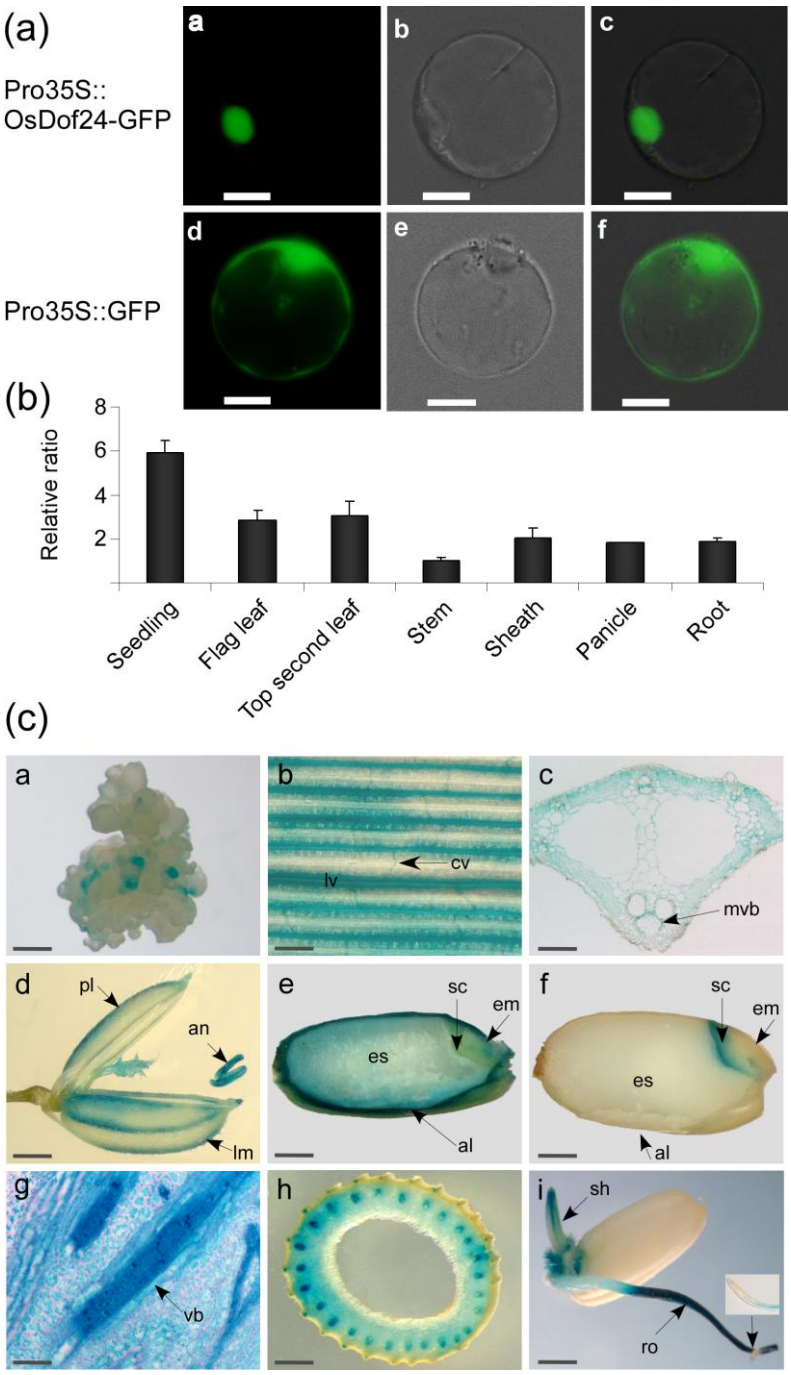

Figure 1. Subcellular localization of a GFP-tagged OsDof24 protein in rice protoplasts, and expression profile of *OsDof24*.

(a) Protoplasts were transiently transformed with either construct Pro35S::OsDof24-GFP (a, b and c) or Pro35S::GFP (d, e and f). After culturing overnight, protoplasts were observed with fluorescence (a, d) and bright field microscopy (b, e). A merged panel of a and b is shown in panel (c), and the combination of d and e is shown in panel (f). Scale bars represent 2 μm.

(b) qPCR analysis of *OsDof24* expression in seven different tissues. Two week old seedlings, 10 DAF (day after flowering) flag leaves, penultimate leaves, stems, sheath, panicles and roots were analyzed for *OsDof24* expression using qPCR. *Ubi* was used as a control for

normalization of cDNA quantity. Bars represent means and standard deviation (n=3 independent qPCRs).
(c) Histochemical localization of *OsDof24* promoter-*GUS* expression in transgenic rice. Embryogenic callus (a), flag leaves (b), cross section of flag leaves (c), immature spikelet (d), seeds at 10 DAF (e), 15 day after flowering seeds (f), longitudinal section of the SAM (g), vascular bundles (g), cross section of stems (h) and after two days of germination (i). al, aleurone layer; an, anther; cv, commissural vein; em, embryo; es, endosperm; lm, lemma; lv, longitudinal vein; mvb, main vascular bundle; pl, palea; sh, shoots; ro, roots; vb, vascular bundle. Scale bars in a, b, d, e, f and i represent 10 mm, in c and g 100 µm and in h 10 µm, respectively.

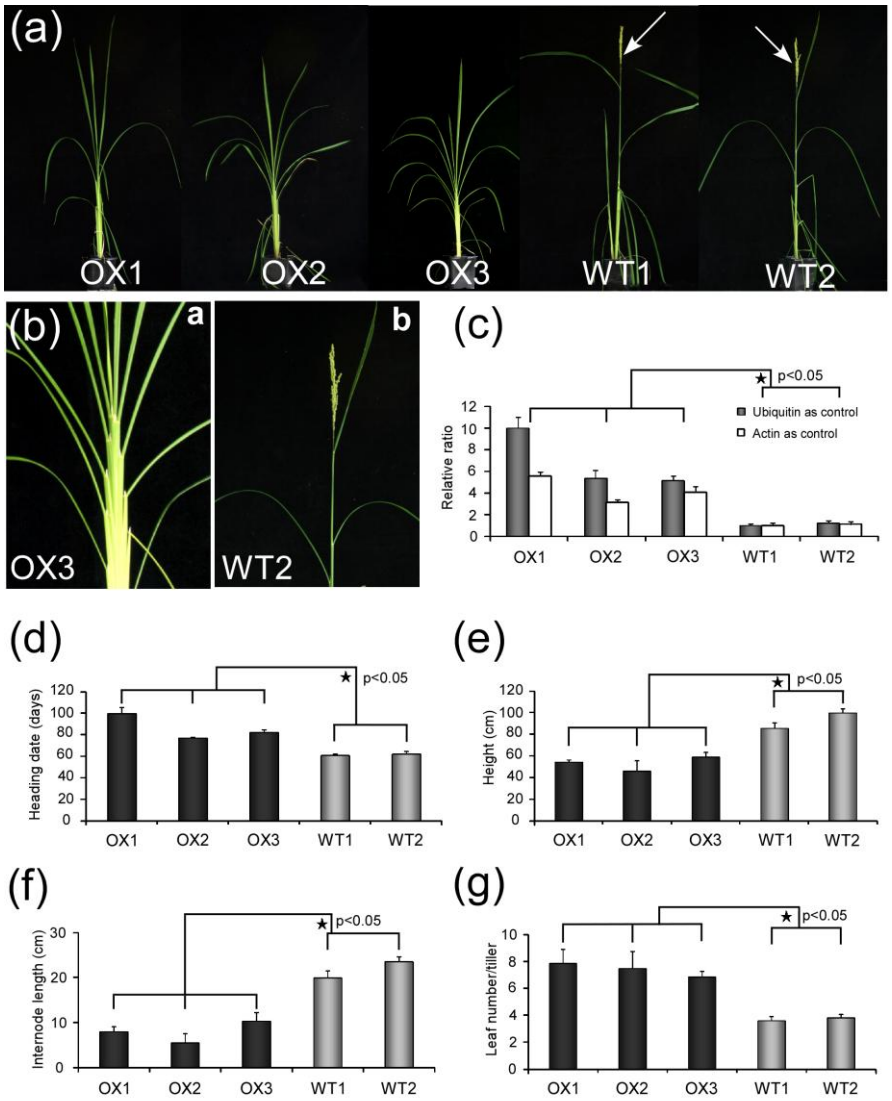

Figure 2. Phenotypical characterization and expression levels of *OsDof24* in overexpression plants and control lines.
(a) Late flowering phenotype of *OsDof24* overexpressing transgenic lines in comparison to control plants. Panicles are indicated by arrows.
(b) Two dimensional leaf distribution and late flowering phenotype of the OsDof24-OX lines. Magnified picture of OsDof24-OX line 3 is shown in panel (left), and a control plant is shown in panel (right).

(c) qPCR results on OsDof24-OX lines 1 to 3. *Ubiquitin* and *Actin* gene expression levels were used as internal controls for normalization of cDNA quantities. Bars represent means and standard deviation (n=3 independent qPCRs).
(d) Flowering time of OsDof24-OX plants and control lines. Flowering time was determined as the number of days from germination (DAG) to the first day that a panicle started flowering.
(e) Height of OsDof24-OX plants and control lines. Height of plants was measured from the soil till the top of the panicle at 120 DAG.
(f) Internode length of OsDof24-OX plants and control lines at 120 DAG.
(g) Average leaf number per tiller of OsDof24-OX plants and control lines at 120 DAG. For each experiment, three or more plants were included. Bars represent means and standard deviation (n ≥3 independent plants measured for each independent transgenic or control line). The data were analyzed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

Plants	Heading date (day)	Height (cm)	Leaf number/tiller	Internode length (cm)
OX1	100.00 ± 5.24	54.57 ± 2.05	7.86 ± 1.02	8.00 ± 1.15
OX2	77.00 ± 0.82	46.00 ± 9.80	7.50 ± 1.22	5.50 ± 2.04
OX3	82.57 ± 2.54	59.00 ± 4.37	6.86 ± 0.40	10.43 ± 1.79
WT1	60.80 ± 1.49	85.80 ± 4.82	3.60 ± 0.32	20.00 ± 1.47
WT2	62.00 ± 2.48	100.00 ± 4.08	3.80 ± 0.26	23.60 ± 1.05

$P<0.05$

Table 1. Overview of phenotyping results from *OsDof24-OX* and wild type plants. Flowering time was calculated from the germination to the day that the first floret opened. Plant height was measured from the soil to the top of the highest panicle at 120 DAG. Internode length and leaf number per tiller were also counted at 120 DAG. From every line, three or more plants were measured. The data were analyzed using ANOVA followed by Bonferroni. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

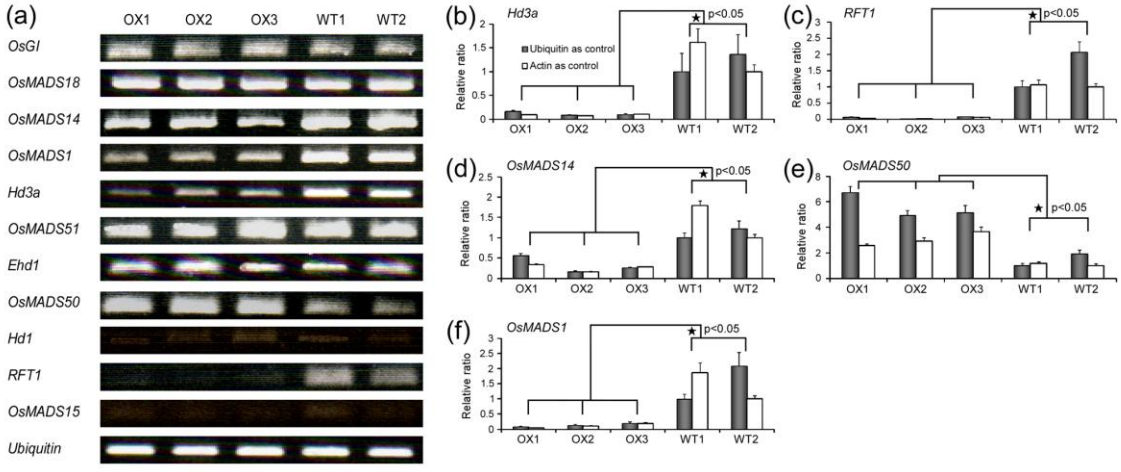

Figure 3. Expression profile of flowering time genes in control and OsDof24-OX plants and qPCR expression profiling of the flowering time genes.

(a) Expression level of *OsGI*, *OsMADS18*, *OsMADS14*, *OsMADS1*, *Hd3a*, *OsMADS51*, *Edh1*, *OsMADS50*, *Hd1*, *RFT1* and *OsMADS15* were analyzed by semi-quantitative RT-PCR. *Ubi* gene expression was used as control for normalization of cDNA quantity.
(b), *RFT1* (c), *OsMADS14* (d), *OsMADS50* (e) and *OsMADS1* (f) in control and OsDof24-OX plants..Both *Ubiquitin* and *Actin* expression levels were used as controls for normalizing cDNA quantities. Vertical bars indicate the standard deviation of three technical replicates for each sample.

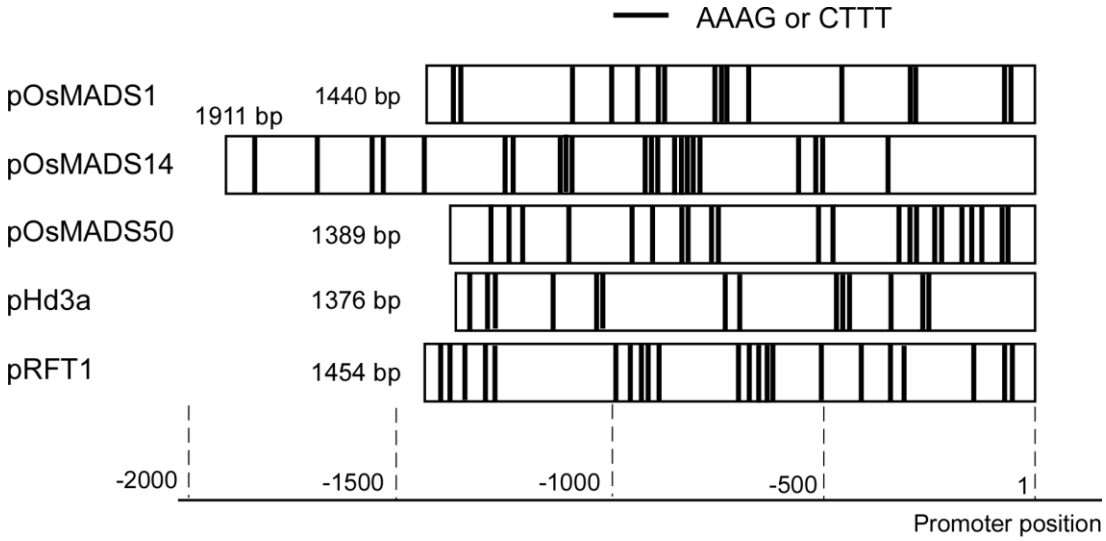

Figure 4. The distribution of the Dof binding motif in the promoters of flowering time-related genes *OsMADS1*, *OsMADS14*, *OsMADS50*, *Hd3a* and *RFT1*.

Downregulation of OsDof24 does not result in phenotypical changes

Given the results obtained with the overexpression plants, we were also interested to see the effect of a loss-of-function approach either using mutants or RNAi lines. We checked available mutant collections in RiceGE (http://signal.salk.edu/cgi-bin/RiceGE) and identified one T-DNA mutant line in *OsDof24* (PEG_3A00724 from the Postech collection (Jeong *et al.* 2006)). BLAST analysis showed that the T-DNA was inserted 640 bp upstream of the ATG. Using Southern blotting, homozygous and heterozygous plants of this mutant were identified. However, homozygous mutant plants did not show any obvious phenotype and we also confirmed that expression was not down-regulated (results not shown). Thus apparently, this mutation did not have any effect on expression. Subsequently, a binary vector construct enabling an RNAi approach was made and transgenic plants were generated. The expression of *OsDof24* was checked and was found down-regulated in two of the transgenic lines although it was still expressed to some level (Figure S2). However, the RNAi plants did not show any obvious phenotypical difference (Figure S3), neither in architecture nor in flowering time.

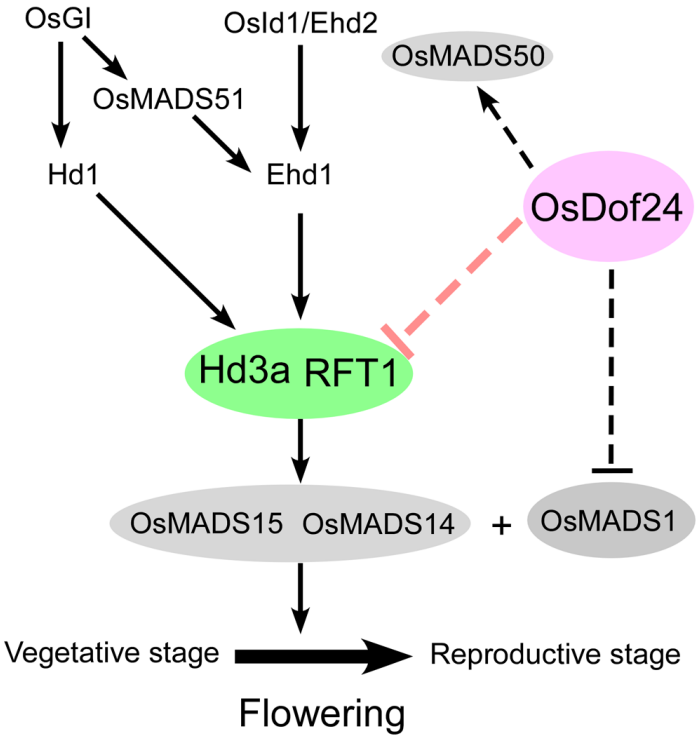

Figure 5. Model of the flowering time pathway under a 12 h light/12 h dark condition in rice. *OsDof24* overexpression down-regulates expression of *OsMADS14*, *OsMADS1*, *Hd3a* and *RFT1*, but does not affect *Hd1* and *Ehd1*. According to this model, *Hd3a* and *RFT1* function as activators which are critical for flowering time and they also regulate expression of *OsMADS14*. *OsDof24* act as a repressor of flowering time by downregulating expression of *Hd3a* and *RFT1*.

Effects of *OsDof24* overexpression on flowering time genes

Genes that have been reported to determine flowering time in rice include *Ehd1, Hd3a, Hd1, RFT1, OsMADS50, OsMADS14, OsMADS15, OsMADS18, OsMASD51, OsMADS1* and *OsGI* and this group will likely increase (Kojima *et al.* 2002; Doi *et al.* 2004; Lee *et al.* 2004; Kim *et al.* 2007; Komiya *et al.* 2008;). To study the link between *OsDof24* and these regulators, we analyzed their expression levels using RT-PCR in flag leaves at 10 DAF in control and *OsDof24-OX* plants. As shown in Figure 3, the expression of *Ehd1*, *Hd1*, *OsMADS18*, *OsMADS15*, *OsMADS51* and *OsGI* were not significantly affected, but the transcription level of *OsMADS50* was upregulated in the *OsDof24-OX* plants, whereas the expression level of *Hd3a*, *OsMADS1*, *OsMADS14* and *RFT1* was down-regulated.

For a subset of flowering time genes, the expression level was further analyzed using qPCR (Figure 3). The results showed that *Hd3a*, *RFT1*, *OsMADS14* and *OsMADS1* were down-regulated in the *OsDof24*-OX plants compared to the control plants, whereas *OsMADS50* was upregulated thereby confirming the RT-PCR

results. All these data suggested that *OsDof24* might influence rice flowering time by regulating the expression of flowering time genes.

Discussion

Dof proteins are plant-specific transcription factors, with known functions as transcriptional activator or repressors playing critical roles during plant growth and development (for a review see Yanagisawa 2004). Among the 30 Dof transcription factors in the rice genome (Lijavetzky *et al.* 2003), so far only *OsDof3* (Washio 2001, 2003) and *OsDof12* (Li *et al.* 2009) have been studied in more detail. In this study, we report on the functional characterization of *OsDof24* and propose a role in regulating flowering time, plant height and leaf growth.

Expression profiling showed that *OsDof24* was expressed in a variety of tissues including two weeks seedlings, 10 DAF leaves, stems, sheaths, panicles and in roots. Furthermore, GUS stainings of transgenic rice containing construct *ProOsDof24::GUS* confirmed expression in seedling and also in leaves, stems, panicles and roots of the plants in filling stage, which is in accordance with the expression profile as determined by qPCR. In addition, we also observed a strong GUS signal in germinating seeds of two days old but we do not find the GUS signal in immature seeds. Like most transcription factors, OsDof24 is targeted to the nucleus in rice. The expression profile of *OsDof24* indicates that it is important in different stages of different tissues during rice growth and development.

To describe the functions of *OsDof24* in more detail, we generated *OsDof24* overexpressing and RNAi rice plants. *OsDof24-OX* plants were two weeks delayed in flowering time under SD condition and are also reduced in plant height. The obvious flowering late and reduced height phenotype indicates that OsDof24 is a repressor of rice heading date under 12 h light/12 h dark condition. In addition, the leaves grew in two dimensions instead of three dimensions. Thus, *OsDof24* is not only a regulator for heading date but also plays an important role in other developmental processes determining plant architecture. We also tried to study the effects of downregulating *OsDof24*, but we did not find an obvious phenotype which could be due to functional redundancy with other Dof genes or simply because the expression level was not downregulated sufficiently.

In rice there are two major flowering time pathways operational, one is controlled by *Hd1*, the other by *Ehd1* (Yano *et al.* 2000; Doi *et al.* 2004). In the *Hd1* pathway, Hd1 promotes rice flowering by activating expression of *Hd3a* under short day (SD) conditions (Yano *et al.* 2000). In the *Ehd1* pathway, *OsMADS51*, which is a B-type response regulator working upstream of Edh1, promotes flowering in the absence of a functional allele of *Hd1*. Ehd1 also promotes flowering by inducing expression of an *FT* like gene under SD condition (Doi *et al.* 2004). Although there are two different pathways, in the end, *Hd3a* and *RFT1* are most critical for the control of the heading date (Kojima *et al.* 2002; Tamaki *et al.* 2007; Komiya *et al*. 2008) and they are also recognized as candidate florigens (Komiya *et al.* 2009). In this study, we

analyzed the expression of 11 flowering time genes in the *OsDof24-OX* lines in order to better understand the function of *OsDof24* in regulating flowering time. In the *OsDof24-OX* lines, *OsMADS50* was found up-regulated, whereas *OsMADS14*, *OsMADS1*, *RFT1* and *Hd3a* were down-regulated under SD condition, Expression of *Hd1*, *EHd1*, *OsMADS51*, *OsGI*, *OsMADS15* and *OsMAD18* was not significantly changed. Using bioinformatics we checked for presence of the Dof binding motif AAAG or its reverse complement sequence CTTT in the promoters of *OsMADS50*, *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1* after cloning them from *indica* rice. The Dof putative binding motif AAAG or its reverse complement sequence CTTT was found in all five promoters with a number varying from 14 to 22. This indicates that the OsDof24 protein might directly interact with the promoters and as a consequence regulate the expression. *OsMADS14* is a downstream gene of *Hd3a* and *RFT1* (Komiya *et al.* 2008). Therefore the downregulation of *Hd3a* and *RFT1* in transgenic plants overexpressing *OsDof24* leads to the downregulation of *OsMADS14*. Thus, overexpression of *OsDof24* specifically affects expression of sets of flowering time genes and it is likely that this takes place on the level of binding of OsDof24 protein to specific binding sites in downstream genes.

Based on previous reports and our results, we propose an updated model to explain the network in regulating heading date in rice (Figure 5). As explained above, heading date in rice is controlled by the *Hd1* and *Ehd1* pathways which share genes. Both *Hd1* and *Ehd1* are upstream of *Hd3a* and *RFT1* (Komiya *et al.* 2009) in the regulatory pathway controlling flowering time (Komiya *et al.* 2008). Hd3a and RFT1 promote flowering by inducing the expression of *OsMADS14* and *OsMADS15*, which interact with the protein OsMADS1 thereby promoting flowering (Lim *et al.* 2000). Normally, the expression of *RFT1* is very low compared to *Hd3a*, and Hd3a promotes rice flowering under SD condition. When *Hd3a* expression was down-regulated by an RNAi approach, *RFT1* became activated to regulate the flowering under SD condition (Komiya *et al.* 2008). Our results indicate that OsDof24 is an important regulator of flowering time by functioning as a repressor of flowering time genes including *Hd3a* and *RFT1*. Overexpression of *OsDof24* results in delayed flowering. *MADS50* was up-regulated in *OsDof24-OX* plants but it acts upstream of *FRT1* under LD (long day) condition (Lee *et al.* 2004; Komiya *et al.* 2009). Different molecular mechanisms controlling plant flowering time under different day length conditions would be the reason that over-expressing *OsDof24* did not promote flowering by up-regulating transcription of *OsMADS50* in the *OsDof24-OX* plants. Taken together, these results suggest that OsDof24 represses flowering by regulating expression of flowering time genes in particular via *Hd3a* and *RFT1* (Komiya *et al.* 2008). Understanding the molecular basis of the activation and repression of *Hd3a* and *RFT1* and the role of *OsDof24* therein will be very helpful for further investigate the mechanisms controlling rice heading date.

Materials and methods

Plants and growth conditions

Transgenic rice seedlings were pre-grown in plant developmental medium (basal ½ MS-medium with 10 g/L sucrose and 2.5 g/L phytagel, adjust ph to 5.8) before transfer to soil and grown under a dark-light cycle of 12h light (28°C, 80% humidity), and at 12 h darkness (21°C, 60% relative humidity) and at a light intensity (photosynthetically active radiation value) of 180 µmol/m2/s.

For protoplast isolation used in transient expression assays, rice seeds were sown into soil directly and grown in the same conditions as above. Flowering time was calculated from the germination to the day that the first flower opened. Plant height was measured from the soil to the top of the highest panicle 120 days after germination (DAG).

RNA isolation, cDNA cloning, RT-PCR and qPCR

Leaves from plants of hybrid rice 2186 (*Oryza sativa* L. ssp. *indica*) were ground in liquid nitrogen and total RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen). Genomic DNA contaminants were removed

from RNA samples by incubating with DNA-free™ (Ambion) at 37°C for 30 minutes. First-strand cDNA was synthesized starting from 1 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) as described by the manufacturer and used in RT-PCR and qPCRs as described below.

The ORF of *OsDof24* was obtained using PCR with primers *OsDof24*-F1 and *OsDof24*-R1 (Table S1a). PCR conditions were five minutes of initial denaturation at 98°C, 36 cycles of denaturation at 98°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 10 min. To confirm the identity of the PCR products, they were first cloned in pCR-blunt II-TOPO vector (Invitrogen) and sequenced commercially (BaseClear, Leiden, The Netherlands).

For analysis of the expression profile of *OsDof24*, two week old seedling and flag leaves at 10 DAF, top second leaves, sheaths, stems, roots and panicles of rice cultivar Minghui 86 were used for RNA isolation and cDNA synthesis by qPCR. To determine the expression level of *OsDof24* and several heading date-related genes, flag leaves from the T_1 generation of *OsDof24*-OX and RNAi lines were used as material. Non-transgenic plants separated from the T_0 generation were used as control.

For qPCR assays, reactions were performed in an optical 96-well plate with an ABI PRISM® 7500 qPCR System (Applied Biosystems). SYBR® Green was used to monitor dsDNA synthesis. All reactions contained 12.5 μl 2× SYBR® Green Master Mix Reagent (Applied Biosystems), 2.0 ng cDNA and 10 pmol of each gene-specific primer in a final volume of 25 μl. Thermal cycling was as follows: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec. Relative expression level of reporter and target genes was determined based on the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen 2001). Both *Ubiquitin* and *Actin* were used as controls for cDNA quantification. Primers used for qPCR are listed in Table S1.

Sub-cellular localization of OsDof24 protein

In order to make a construct for subcellular localization studies, the ORF of *OsDof24* was PCR-amplified with primers *OsDof24*-F5 and *OsDof24*-R5 (Table S1a), and cloned as *Xho*I/*Nco*I fragment into vector pTH2 (Chiu *et al.* 1996) between *Sal*I and *Nco*I. The resulting construct Pro35S::OsDof24-GFP carried the ORF of *OsDof24* in frame to the N-terminus of the green fluorescent protein (GFP) gene, was used in protoplast transformation. The empty vector pTH2 containing a *GFP* gene (with S65T mutation) driven by the CaMV 35S promoter was used as a control. Protoplast transformations were performed as described by Chen *et al.* (2006). After transformation, the protoplasts were incubated at 28°C overnight in K3 buffer and then observed using Confocal Laser Scanning Microscopy (CLSM) using excitation and emission filters Ex450-490 and BA520-560 for GFP.

Binary vector construction and rice transformation

For fusing the *OsDof24* promoter with the *GUS* gene, a 2,405 bp fragment upstream of the predicted translation start site was amplified from cultivar Minghui 86 using Phusion polymerase (Finnzyme) using primers OsDof24F and OsDof24R (Table S1d). The PCR fragment was subsequently cloned into vector pCR2.1 Topo (Invitrogen), sequence verified (BaseClear, Leiden, The Netherlands) and then inserted into vector pCAMBIA-1391Z (GenBank Accession AF234312) with *Eco*RI/*Nco*I sites, generating construct *ProOsDof24::GUS*.

For constructing an *OsDof24* overexpressing vector, the full length sequence of *OsDof24* was PCR-amplified with primers *OsDof24*-F2 and *OsDof24*-R2 (Table S1) and then digested with *Nco*I/*Bam*HI. The excised fragment was cloned using the same sites between the *GOS2* promoter and the *nos* terminator sequence in binary vector pCAMBIA-1300intC (GenBank Accession AF294978).

To down-regulate expression of *OsDof24* using an RNAi approach a fragment of the gene was obtained by PCR and then inserted into pHANNIBAL vector first (Wesley *et al.* 2001). The antisense fragment of *OsDof24* was generated using primers OsDof24-F3 and OsDof24-R3 (Table S1a), then digested by *Cla*I/*Bam*HI and finally inserted into pHANNIBAL using the same sites. The sense fragment of *OsDof24* was generated using primers *OsDof24*-F4 and *OsDof24*-R4 (Table S1a) and then excised with *Xho*I/*Kpn*I and finally inserted into pHANNIBAL carrying the antisense fragment. This plasmid was digested with *Sal*I/*Spe*I and the resulting fragment was inserted into vector pCAMBIA-1300intC cut with *Sal*I/*Xba*I. The resulting binary vector was used in rice transformation.

Rice transformation of *japonica* cultivar Zhonghua 11 was performed as described by Scarpella *et al*. (2000) instead that *A. tumefaciens* LBA4404 was used for transformation. Prior to growth in the greenhouse, transgenic seedlings were selected on a half-strength Murashige-Skoog medium supplied with 0.7% type I agarose (Sigma) and 25 µg/ml hygromycin. Transgenic lines were further characterised by Southern blotting using the *hpt* gene as a probe to determine copy number. Single copy lines were selected for further analysis.

Detection of GUS expression, cytological techniques and microscopy

Histochemical detection of GUS activity, cytological techniques and microscopy were performed as described earlier (Samach *et al.* 2000). Samples were viewed using a Leica MZ12 stereo microscope or a Leitz Diaplan microscope with bright-field optics settings and images were acquired with a Sony 3CCD Digital Photo Camera DKC-5000.

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Supplemental data

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    ATGCAGGAGCAGCAGCCGGAGACCGGCCGCCGGCCGGCGCAGCAGTTCGCCACCGTCGAC  60
1     M  Q  E  Q  Q  P  E  T  G  R  R  P  A  Q  Q  F  A  T  V  D

    CTGCGCCGTCCCAAGGGCTACGCGGCGGCTCCCGCGACGCCACAGCCTGGTTCGGCTGCG 120
21    L  R  R  P  K  G  Y  A  A  A  P  A  T  P  Q  P  G  S  A  A

    ACTGCTGCTGCCGCCGCTGGCCCGGCTGCGACGGCGGCAGCGGCGGCGGCGGGGGAGGGT 180
41    T  A  A  A  A  A  G  P  A  A  T  A  A  A  A  A  A  G  E  G

    GACCCGTGCCCGCGGTGCGAGTCGCGGGACACCAAATTCTGCTACTACAACAACTACAAC 240
61    D  P  C  P  R  C  E  S  R  D  T  K  F  C  Y  Y  N  N  Y  N
             ★        ★
    ACCTCCCAGCCCCGGCACTTCTGCAAGTGTTGCCGCCGCTACTGGACCAAGGGTGGCACG 300
81    T  S  Q  P  R  H  F  C  K  C  C  R  R  Y  W  T  K  G  G  T
                            ★        ★
    CTCCGCAACGTCCCCGTCGGCGGCGGCACGCGCAAGAAGTCCTCGTCTTCGTCGTCGTCG 360
101   L  R  N  V  P  V  G  G  G  T  R  K  K  S  S  S  S  S  S  S

    TCTTCCTCGTCATCCGCCGCCGCCGCAGCACCCGCCGCCAAGCGCCAGAAGACGTCGAAG 420
121   S  S  S  S  S  A  A  A  A  A  P  A  A  K  R  Q  K  T  S  K

    AAGCGCCGCGTCACGACTCCCGAGCCCCTCGCCGCCACCACCCCCGTCCTCACCGAAGCC 480
141   K  R  R  V  T  T  P  E  P  L  A  A  T  T  P  V  L  T  E  A

    GCCGCTGACTCCGCCGCCAAGACGACGACCGAAGCTACGTCGGAGAAGAAGACGACGACT 540
161   A  D  S  A  A  K  T  T  T  E  A  T  S  E  K  K  T  T  T

    TCCACAACGACGACGACACCGCCGGCGCCTGACACCACGAGCGAGATCACCACGGAGCTC 600
181   S  T  T  T  T  T  P  P  A  P  D  T  T  S  E  I  T  T  E  L

    GTCGTCCCGGCCGTGGAGGAGGACTCGTTCACGGACCTCCTGCAGCCGGACTCCGCCGCC 660
201   V  P  A  V  E  E  D  S  F  T  D  L  L  Q  P  D  S  A  A

    GTCACCCTCGGCCTCGACTTCTCCGACTACCCGTCCATCACCAAGAGTCTGGCCGACCCG 720
221   V  T  L  G  L  D  F  S  D  Y  P  S  I  T  K  S  L  A  D  P

    GACCTGCACTTCGAGTGGCCGCCGCCGGCGTTCGACATGGCGTCCTACTGGCCGGCCGGC 780
241   D  L  H  F  E  W  P  P  P  A  F  D  M  A  S  Y  W  P  A  G

    GCCGGGTTCGCCGACCCGGACCCGACCGCCGTGTTCCTCAACCTCCCATGA          831
261   A  G  F  A  D  P  D  P  T  A  V  F  L  N  L  P  *
```

Figure. S1 Nucleotide sequence and predicted amino acid sequence of the *OsDof24* gene (LOC_Os08g38220). The Dof domain in the N-terminus is indicated by underlines and the four cysteine residues which are characteristic for the Dof zinc finger of *OsDof24* are marked by stars.

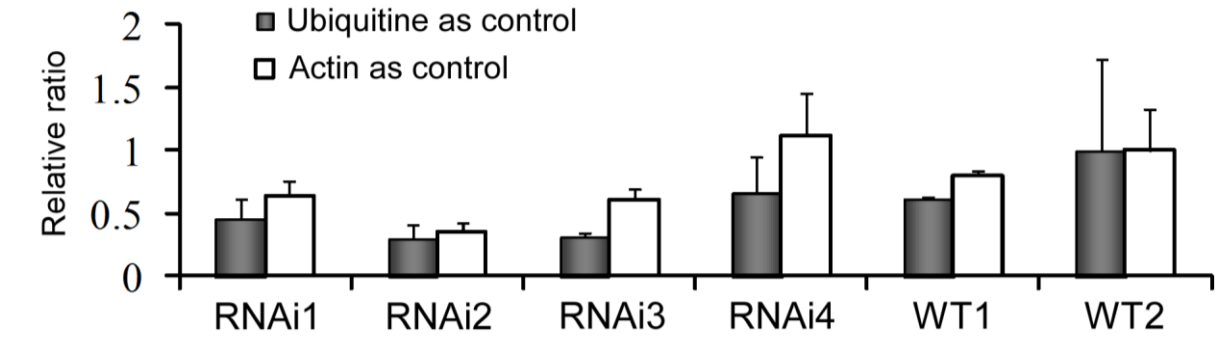

Figure. S2 Expression levels of *OsDof24* in the RNAi transgenic plants. *Ubiquitin* and *Actin* gene expression levels were used as internal controls for normalization of cDNA quantities. Bars represent means and standard deviations (n=3 independent qPCRs).

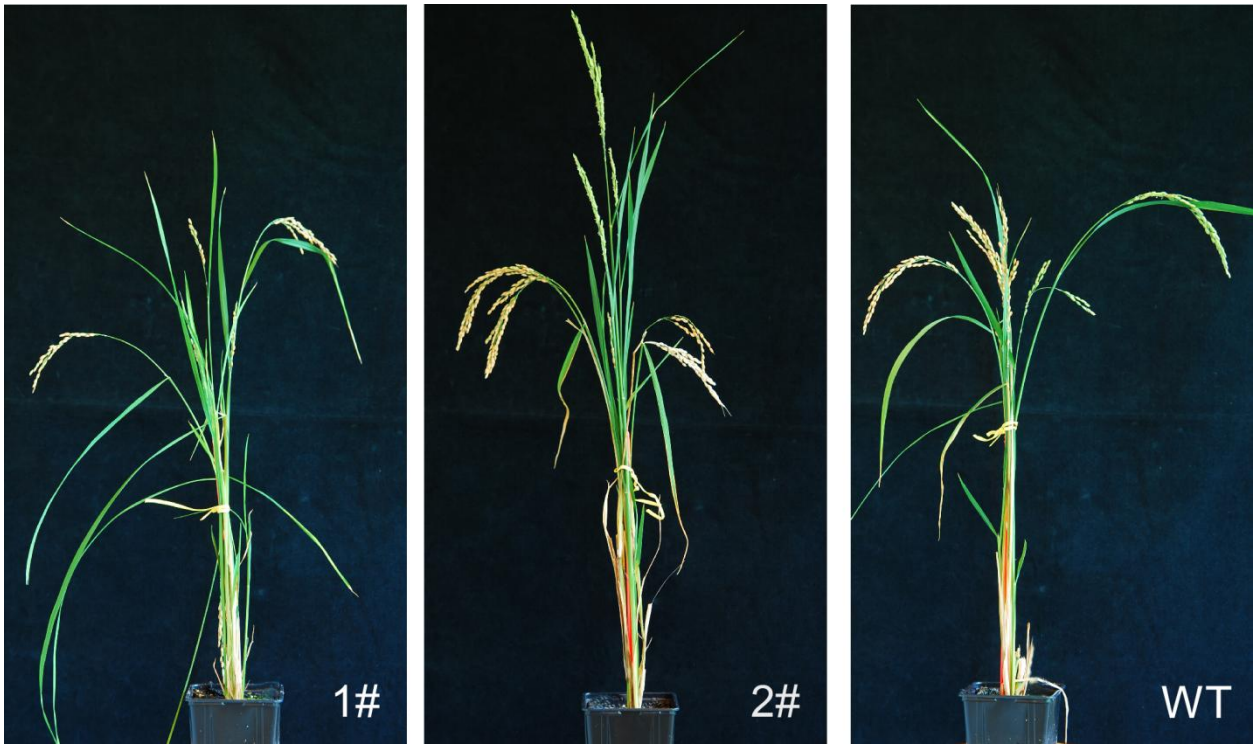

Figure. S3 Downregulation of *OsDof24* using transgenic RNAi lines 1 and 2 from Fig. S2 does not result in phenotypical changes. The results of two independent *OsDof24* RNAi transgenic plants and one control plant are shown.

Table S1 Primer sequences used for gene cloning, RT-PCR, real time quantitative PCR and cloning of the *OsDof24* promoter (LOC_Os08g38220).

Table S1a Primers used for *OsDof24* cDNA cloning and vector construction

Primer name	Primer Sequences (5' to 3')	Restriction enzyme site
OsDof24-F1	CGGGATCCATGCAGGAGCAGCAGCCGGA	*Bam*HI
OsDof24-R1	GGAATTCTCATGGGAGGTTGAGGAACACGGC	*Eco*RI
OsDof24-F2	CATGCCATGGAGGAGCAGCAGCCGGAG	*Nco*I
OsDof24-R2	CGGGATCCTCATGGGAGGTTGAGGAAC	*Bam*HI
OsDof24-F3	CCATCGATGCTGCCGCCGTCGCAG	*Cla*I
OsDof24-R3	CGGGATCCAGCAGCCGGAGACCG	*Bam*HI
OsDof24-F4	CCGCTCGAGAGCAGCCGGAGACC	*Xho*I
OsDof24-R4	GGGGTACCGCTGCCGCCGTCGCAG	*Kpn*I
OsDof24-F5	CCGCTCGAGATGCAGGAGCAGCAGCCGGAG	*Xho*I
OsDof24-R5	CATGCCATGGATGGGAGGTTGAGGAACACG	*Nco*I

Table S1b Primers used for RT-PCR

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
Hd3a	ATGGCCGGAAGTGGCAGGGAC	ATCGATCGGGATCATCGTTAG
Hd1	TTCTCCTCTCCAAAGATTCC	CATACGCCTTTCTTGTTTCA
Ehd1	GTTGCCAGTCATCTGCAGAA	GGATGTGGATCATGAGACAT
RFT1	CAAGGATCGTCTCCAATG	ACCAGCCAGTGTAGATAC
OsMADS51	GTTTGCTCTGCTCCTACTC	ACTCCTCCTCCAGCATTGAA
OsMADS14	TCCTATGCAGAAAAGGTCCTT	GGACGAAGCCAAAATATACAC
OsMADS15	CGTCGTCGGCCAAACAG	TGACTTCAATTCATTCAAGGTTGCT
OsMADS50	CAGGCCAGGAATAAGCTGGAT	TTAGGATGGTTTGGTGTCATTGC
OsGI	TGGAGAAAGGTTGTGGATGC	GATAGACGGCACTTCAGCAGAT
OsMADS18	CCAAACTGGATGCACTTCAG	ATCAATATCGCTGGAAGATG
OsMADS1	TCCATATGTCCTGGCAAGAT	AAGAGAGCACGCACGTACTT
Ubiquitin	AGCAGAAGCACAAGCACAAG	AAGCCTGCTGGTTGTAGACG

Table S1c Primers used for qPCR

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
OsDof24	ACGAGCGAGATCACCA	GGTCGGCCAGACTCTT
Hd3a	CACCTATGGCTCCAAGAC	GACCAACCAATGTAGATACTC
OsMADS14	CGGTTGCGAGACGAGGAA	GAAAGACGGTGCTGGACGAA
OsMADS50	CAGGCCAGGAATAAGCTGGAT	TTAGGATGGTTTGGTGTCATTGC
RFT1	CAAGGATCGTCTCCAATG	ACCAGCCAGTGTAGATAC
OsMADS1	TCCATATGTCCTGGCAAGAT	AAGAGAGCACGCACGTACTT
Ubiquitin	AGCAGAAGCACAAGCACAAG	AAGCCTGCTGGTTGTAGACG
Actin	GACCCAGATCATGTTTGAGACC	CATCACCAGAGTCCAACACAATAC

Table S1d Primers used for cloning of promoter constructs

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
OsMADS14	GGATCCAGAGCTATGATGGAGGCTCG	GAATTCCTTCCTCCTGTTCTTCCTCC
Hd3a	GGATCCGTAACATATTTGCCACCTATTG	CCATGGCGATCTTGCAAAAAACCCTG
OsMADS50	CGGGATCCGGAGTAGTACTTACTAGTGC	CATGCCATGGGAACCAACCAACCACACGAG
RTF1	CGGGATCCGCAAGTCGATCTGGAAGCTAG	CATGCCATGGGTCAAATTAATAACCTCTAAC
OsMADS1	CGGGATCCCAACACGAGGAAGAGGCAACAC	CATGCCATGGCTTCTTCCTCCTCCTCTC
OsDof24	CAGGTACTATAGCAGGCTATAAGGCCGC	CCATGGTGGCTGCTCTGGTCCCTCCGCGCG

Chapter 3

Functions of OsDof25 in regulation of *OsC4PPDK*

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Running title: Role of OsDof25 in regulation of *OsC4PPDK*

Four key words: Dof, OsC4PPDK, transcription factor, rice

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Abstract

Relative little is known about the functions of the so-called Dof zinc factors in plants. Here we report on the analysis of OsDof25 and show a function in regulation of the important C4 photosynthesis gene, *OsC4PPDK* in rice. Over-expression of *OsDof25* enhanced the expression of *OsC4PPDK* in transient expression experiments by binding in a specific way to a conserved Dof binding site which was confirmed by yeast and *in vitro* binding studies. Expression studies using promoter GUS plants as well as qPCR experiments showed that *OsDof25* expressed in different tissues including both photosynthetic and non-photosynthetic organs and that expression of OsDof25 was partially overlapping with the *OsC4PPDK* gene. Conclusive evidence for a role of OsDof25 in regulation of *C4PPDK* came from loss-of-function and gain-of-function experiments with transgenic rice, which showed that down-regulation or over-expression of *OsDof25* correlated with *OsC4PPDK* expression and that OsDof25 has functions as transcriptional activator.

Introduction

Rice (*Oryza sativa L*.) is one of the most important food crops in the world because it feeds more than half of global population and is daily carbohydrate source in many countries. Traditional breeding only results in an annual yield increase of about 1% whereas this should be higher to keep in pace with the increase in demand. Much is expected from molecular breeding as a tool to combine the best genes and alleles in novel plant types but to enable this we need new information about which genes and alleles are responsible for which traits. To enable this process, many national and international genetics and genomics projects on rice were initiated in the last decade and as results the genomes of indica and japonica have been completely or partially sequenced (Matsumoto et al. 2005), a large number of T-DNA insertion or transposon-based tagged mutants have been produced (Hirochika et al. 2004; Jung and An 2013; Priya and Jain 2013), genome wide expression profiles have been obtained by using microarray and SAGE (Bao et al. 2005; Li et al. 2006). All these approaches have provided very useful information and resources enabling functional genomics with rice. However, the function of the majority of rice genes has not been experimentally demonstrated (Zhang et al. 2006). In particular knowledge on functions of transcription factors and other regulatory proteins could be relevant for improvement of rice since some of them are related to important traits in cereals. Examples of such transcription factors are Opaque2 from maize that is a regulator of seed-storage protein deposition and TB1 which plays a major role in tiller number and apical dominance in maize (Hwang et al. 2004). In rice, MOC1 was shown to be important in determining tiller number (Li et al. 2003) and OsSPL14 was shown to be involved in tiller number and grain per panicle number (Luo et al. 2012).

Transcription factors play key roles in regulating gene expression at the transcriptional level. According to the rice transcription factors database, there are at

least 63 transcription factor families in the rice genome (Gao et al. 2006). One of them is the Dof gene family (for reviews see Yanagisawa 2002; Noguero et al. 2013) which has important functions in responses to plant hormones such as gibberellin (Washio 2001; Mena et al. 2002) and auxin (DePaolis et al. 1996; Kisu et al. 1998), stress responses (Zhang et al. 1995), flowering time (Li et al. 2009; Corrales et al. 2014), tissue specific expression (Yanagisawa 1998; Plesch et al. 2001) and photosynthesis (Yanagisawa and Sheen 1998; Yanagisawa 2000). Dof transcription factors have one copy of the Dof zinc finger domain (hence the name of DNA binding with only One Finger), which normally resides in the N-terminal region, but the sequences outside the Dof domains are very diverse (Riechmann and Ratcliffe 2000; Yanagisawa 1998, 2002). Most of the Dof domain proteins recognise an AAAG motif or the revise complement CTTT as core sequence element in DNA binding assays *in vitro* (Yanagisawa 2002), except for AOBP, a protein from pumpkin, which recognises an AGTA repeat as core binding sequence (Kisu et al. 1998).

ZmDof1 was found to be associated with expression of multiple genes involved in carbon fixation in maize (Yanagisawa 2000), and was shown to be able to activate expression of the *OsC4PPDK* promoter by binding to the AAAG motif (Yanagisawa 2000). OsC4PPDK (pyruvate orthophosphate dikinase) catalyses the conversion of pyruvate into phosphoenolpyruvate (Edwards et al. 1985). This is one of the key steps in the C4 photosynthesis pathway, since PPDK can regenerate the primary CO_2 acceptor phosphoenolpyruvate (PEP) which is the substrate for an important step in carbon fixation in mesophyll cell chloroplasts (Chollet et al. 1996). Since carbon fixation is the most important process underlying yield in cereals, we wanted to study whether Dof transcription factors can be used as a tool to modify *OsC4PPDK* expression. As a first step, we cloned *OsDof25* from rice which together with *OsDof24* is the closest homolog to *ZmDof1* on the protein level and investigated its function in more detail using loss-of-function and gain-of-function studies supported by *in vitro* binding studies. The results show novel insight in regulation of *OsC4PPDK* by OsDof25 and will ultimately contribute to understanding the regulation of C3 and C4 photosynthesis genes in rice.

Results

Phylogenetic analysis of the japonica rice Dof transcription factor family and identification of *OsDof24* and *OsDof25* as closest homologs of *ZmDof1*

A bioinformatics analysis of the japonica rice genome identified 30 Dof transcription factor genes (*Oryza sativa*) (Lijavetzky et al. 2003). All Dof transcription factor genes have been assigned to a chromosomal map position (Fig. 1, Table S1) which is based on the TIGR database (http://plntfdb.bio.uni-potsdam.de/v3.0/) (Ouyang et al. 2007). To determine if there are any paralogous gene pairs we checked their chromosomal locations in relation to the history of genome duplications (Yu et al. 2005). The chromosomal map presented in Fig. 1 shows that the rice Dof genes are not generally clustered and that the pair *OsDof7, -8, -9/-18* and the pair *OsDof24/-25*

are very likely paralogues because they are located in duplicated regions on chromosomes 2/4 and 8/9 respectively.

Alignments of the amino acid sequences of the Dof domains of all 30 Dof proteins, which were downloaded from the transcription factor database (Riano-Pachon et al. 2007), showed that the Dof domains were highly conserved but that outside of the Dof domain there is little conservation (Yanagisawa 2002). Next, we performed a phylogenetic analysis with the rice Dof family and ZmDof1. As shown in Fig. 2, there are two Dof proteins from rice, OsDof24 and OsDof25, in the same clade with ZmDof1. Given the role of ZmDof1 in regulation of the photosynthesis genes *PEPC* and *C4PPDK*, it may very well be that OsDof25 is also involved in regulation of *OsC4PPDK*.

In order to further study the functions of *OsDof25*, this gene was cloned from the Chinese super-hybrid rice combination Liangyou 2186 (GenBank Accession KC996733). Sequence analysis showed that there are two extra serine residues on position 97 compared with the sequence downloaded from the plant transcription factor database (http://plntfdb.bio.uni-potsdam.de/v3.0/). Likely these two serines do not have an serious effect on functionality of the Dof domain of OsDof25 since they are outside this domain (data not shown).

OsDof 25 is an activator of the *OsC4PPDK* promoter

To determine whether OsDof25 can regulate the expression of *OsC4PPDK* an experimental set-up was designed based on transient expression assays in rice protoplasts using OsDof25 effector constructs and *OsC4PPDK* promoter reporter constructs. For this, a 2.5 kb promoter fragment of *OsC4PPDK* was cloned from rice cultivar SE21S and Minghui86 (*Oryza sativa* L. ssp. *indica*) which are the parents of hybrid rice combination Liangyou 2186. Sequencing and alignments showed that there is no difference between the *OsC4PPDK* promoters from cultivars SE21, Minghui 86 (GenBank Accession KC996732) and Nipponbare. The reporter *ProPPDK::GUS* was constructed by introducing a 2.5 kb *OsC4PPDK* promoter fragment fused to the GUS reporter gene as a transcriptional fusion. Plasmid Pro35S::OsDof25, carrying the ORF of *OsDof25* under the control of the *CaMV 35S* promoter was used as effector. As shown in Fig. 3, a combination of this reporter and effector resulted in a doubled GUS activity compared to the empty effector plasmid pRT100. Thus the results strongly suggested that OsDof25 can activate expression of the *OsC4PPDK* promoter *in vivo*.

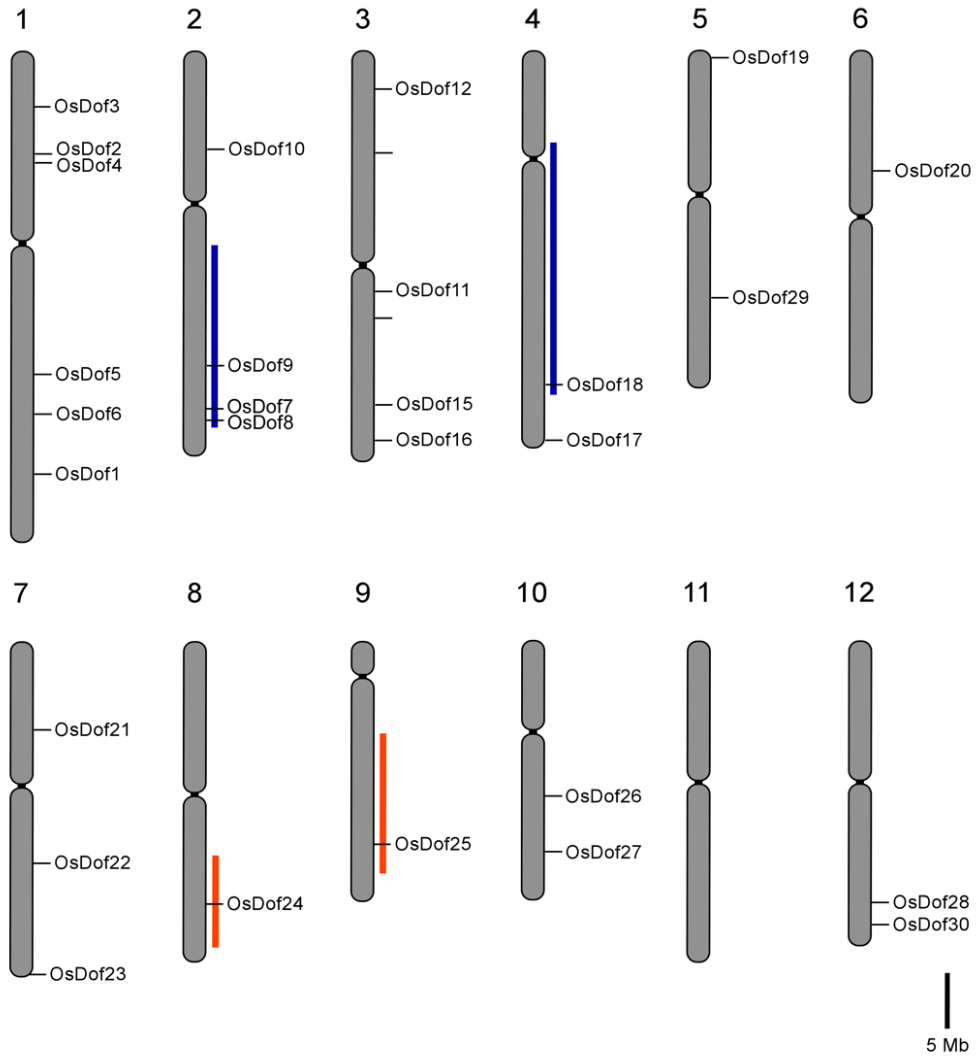

Figure 1. Chromosomal distribution of all Dof transcription factors on the 12 rice chromosomes.
Duplicated regions basing on the whole-genome duplication (Yu et al. 2005) are indicated with different colours. Scale bar is 5 Mb.

It has been reported that most of Dof transcription factors will recognise the sequence AAAG or its reverse complementary sequence, CTTT, as an essential DNA binding motif (Yanagisawa and Izui 1993; Yanagisawa and Schmidt 1999; Yanagisawa 2002). We used PLACE (Higo et al. 1998) to search for *cis*-acting elements, in the 2.5 kb *OsC4PPDK* promoter fragment, and as a result 16 putative binding motifs (AAAG or CTTT) were identified which are indicated in Fig. 3a. To further study the function of the putative Dof binding sites identified in the *PPDK* promoter, a loss-of-function experiment was designed based on a series of five deletion constructs (ProPPDK-Δ1::GUS to ProPPDK-Δ5::GUS) fused to the GUS reporter gene (Fig 3a). As shown in Fig 3b, only co-transfection with reporter ProPPDK-Δ5::GUS and effector Pro35S::OsDof25 did not result in an increase of relative GUS activity, but co-transfection with the other reporters showed a similar

increase of relative GUS activity compared to the full length *OsC4PPDK* promoter. The difference between ProPPDK-Δ4::GUS and ProPPDK-Δ5::GUS is that there is a 111 bp deletion from -385 to -274, which contains one putative Dof binding motif with the consensus sequence CTTT (Fig. 3a) which in principle could be binding site for the OsDof25 protein. Taken together, the results showed that OsDof25 can activate a series of *OsC4PPDK* promoter deletion constructs. Up to coordinate -385, deletion had little effect on activation by OsDof25. In turn, when a 111 bp fragment spanning from -385 to -274 bp containing a putative Dof binding motif was deleted, activation by OsDof25 decreased from two-fold to only one-fold.

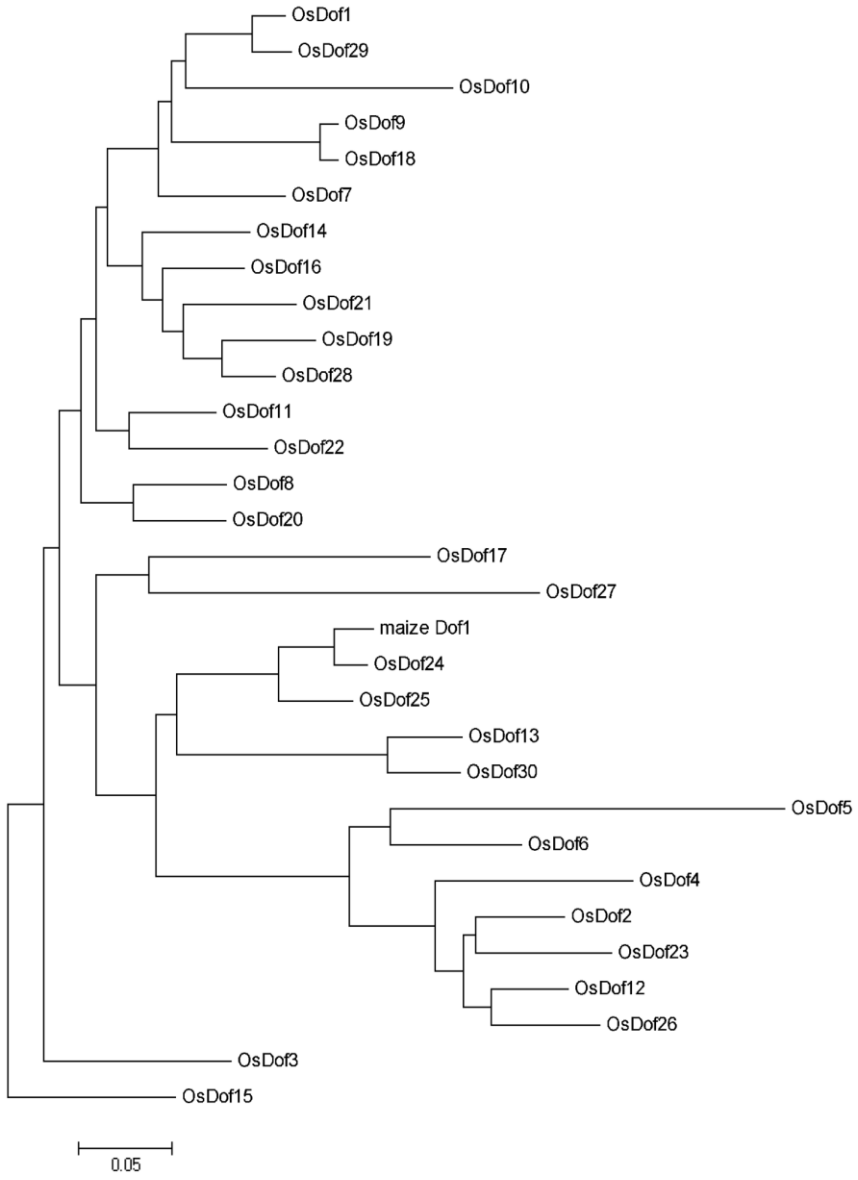

Figure 2. Phylogenetic tree analysis of *ZmDof1* and rice Dof transcription factors proteins. The amino acid residues of the entire Dof transcription factor family were aligned using ClustalX and the tree was created using MEGA4 software.

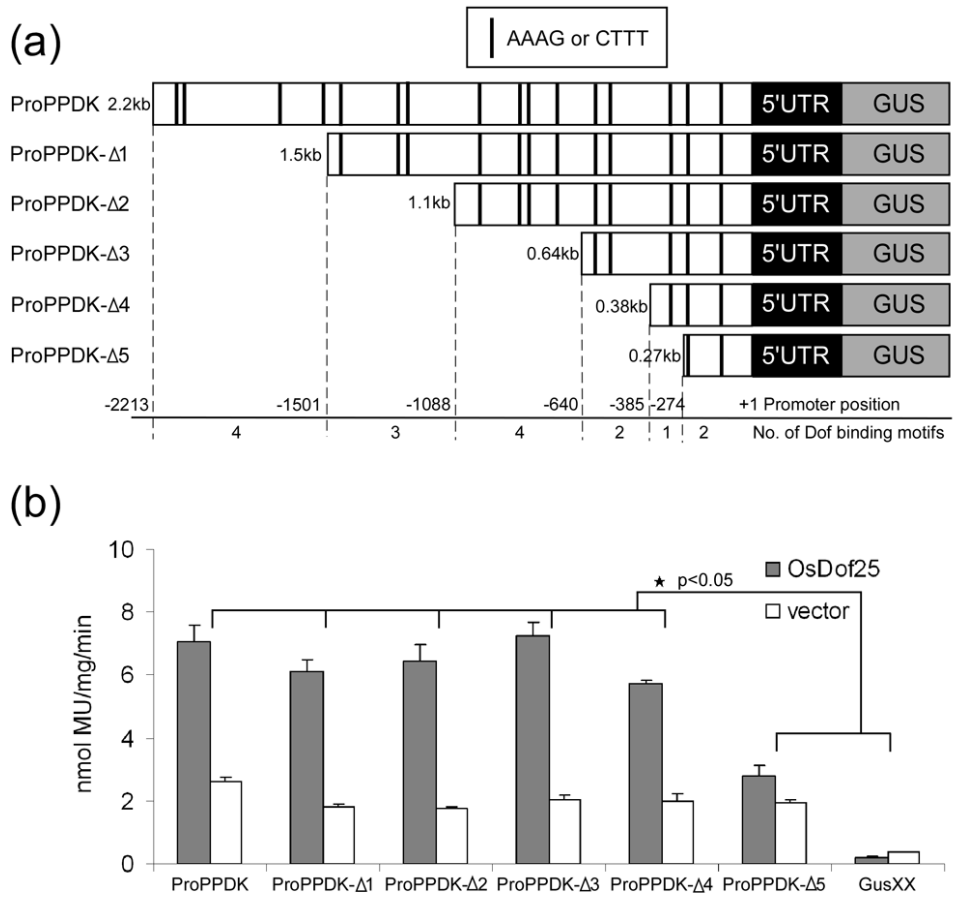

Figure 3. Loss-of-function analysis of the *OsC4PPDK* promoter in rice protoplasts.
(**a**) Schematic representation of truncated promoter-GUS constructs that were used in transient expression assays with rice protoplasts. Promoter lengths are indicated on the left. Negative numerals indicate the nucleotide position relative to the transcriptional start site. Putative Dof binding sites, AAAG or CTTT, as predicted by the PLACE database are indicated by black bars. Numbers of the putative binding motif within truncated regions are indicated.
(**b**) The effects of OsDof25 overexpression and the mapping of OsDof25-binding fragments on the *OsC4PPDK* promoter were tested using an overexpression construct Pro35S::OsDof25 which was co-transformed into rice protoplasts with a series of *OsC4PPDK* promoter GUS constructs. The empty effector plasmid pRT100 was used as a control. Relative GUS activities were normalised for total protein. GUS activities of co-transformation with Pro35S::OsDof25 are indicated in black and columns representing empty effector plasmids are blank. The bar graphs are based on the mean values of three independent transformations of each construct combination and error bars represent means and standard deviations (SD) of biological replicates. The data were analysed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

Although it seems that this particular region in the *OsC4PPDK* promoter is important in regulation we do not exclude that there are important elements further upstream in

the promoter which also contribute to expression together with the region from -385 to -274 bp.

OsDof25 recognises the *OsC4PPDK* promoter through a Dof binding motif

The transient experiments assays indicated that OsDof25 is able to activate the *OsC4PPDK* promoter through a 111 bp fragment (-385 to -274) containing a putative Dof protein binding site. To confirm whether OsDof25 regulates the *OsC4PPDK* promoter by interacting directly with this specific sequence element, a series of Electro Mobility Shift Assays (EMSAs) were conducted. For this, OsDof25 was expressed and purified from *E. coli* as recombinant protein. Next, three different parts of the fragment from -385 to -274 of *OsC4PPDK* promoter with or without the CTTT motif were used as probes (Table S2). In order to determine the specificity of the interaction of OsDof25 protein with the Dof binding motif, a set of four different mutant oligonucleotides was designed and used as EMSA probes (Fig. 4a, Table S2). The EMSA assay of OsDof25 protein with wild type probe P3 produced a distinct complex (Fig. 4b) which could be competed away with a range of unlabelled P3 oligonucleotide (Fig. 4c). On the other hand, when OsDof25 protein was incubated with mutant probes P4, P5, P6 and P7 or two different wild type probes derived from the *OsC4PPDK* promoter (stretching from -385 to -274) without a Dof binding site, than the protein-DNA complex did not appear (Fig. 4b). In conclusion, the EMSAs confirmed that OsDof25 is indeed able to interact specifically with the *OsC4PPDK* promoter through the Dof binding site at position -291 bp.

Furthermore, we also studied the interaction between OsDof25 and the *OsC4PPDK* promoter using a yeast one-hybrid system. For this, probes P3 and the mutant P6 (Table S2), were inserted upstream of the reporter gene *HIS3* in the integrative vector pINT1-HIS3NB (Meijer et al. 1998; Ouwerkerk and Meijer 2001) which was transformed into yeast resulting in strains Y187:ProOsC4PPDK-WT and Y187:ProOsC4PPDK-MU. *OsDof25* was expressed in yeast using a GAL4 AD vector. Because of the fusion with the GAL4 Activation Domain (AD), the resulting vector (pACTIIa-OsDof25) can be used for detection of DNA binding of OsDof25 protein to a target sequence without OsDof25 having its own activation domain. Detection of such interaction is via activation of a *HIS3* reporter gene on yeast cells growing on a minimal medium lacking histidine but containing a minimal concentration (5 mM) of 3-AT which is a competitive inhibitor of His3p activity. Fig. 5a, shows the growth results of all four combinations of effector and reporters on histidine-containing medium as control to show that none of the effectors has any negative effects on growth. As shown in Fig. 5b, the combination of pACTIIa-OsDof25 and the reporter preceded by the *OsC4PPDK* promoter fragment grew well on minimal medium lacking histidine (Fig. 5b) whereas pACTIIa as empty control did not result in any growth. However, the strain with mutant fragment P6 did not grow on the same minimal medium in combination with either pACTIIa-OsDof25 or pACTIIa (Fig 5b). Thus, the results from the yeast one-hybrid system show that OsDof25 indeed

recognizes the -285 to -274 fragment of *OsC4PPDK* promoter which has a putative Dof binding motif. The specificity of OsDof25 is demonstrated by the lack of activation of a mutant fragment where a sequence CTTT was converted into CTAT.

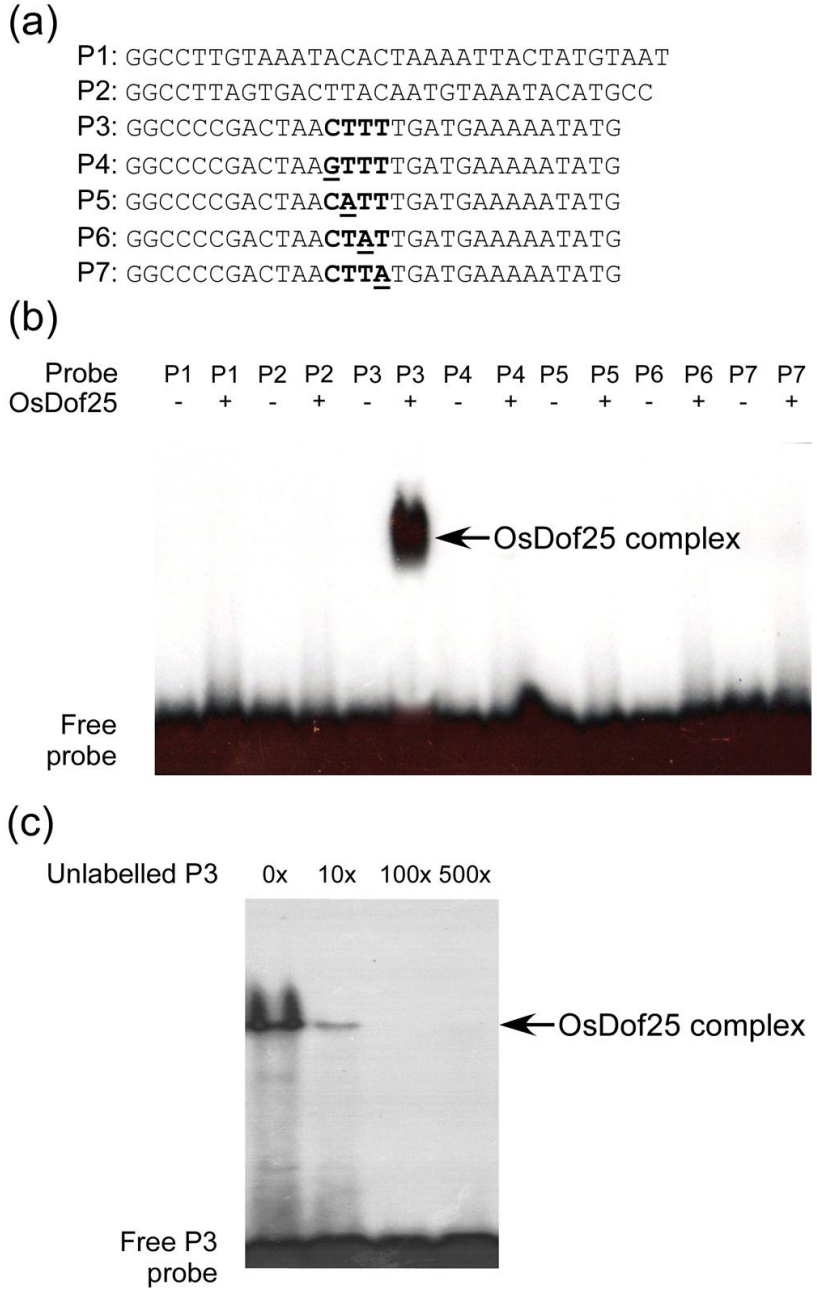

Figure 4. Electrophoretic mobility shift assays (EMSA) of recombinant OsDof25 proteins with oligonucleotides derived from *OsC4PPDK* promoter containing the CTTT motif.
(a) Overview of the probes used in EMSAs. P1 and P2 represent two wild type probes without the putative Dof binding site in the *OsC4PPDK* promoter. The wild type probe with the putative Dof binding motif CTTT (P3) and its mutants are also shown. The CTTT motif is shown by bold letters, and its mutants are shown by underlines.
(b) The ^{32}P-labeled DNA probes were incubated without (-) or with (+) recombinant GST-OsDof25 fusion protein prior to loading on native polyacrylamide gels.
(c) OsDof25 protein binds to probe P3 derived from the *OsC4PPDK* promoter. Competitor fragments were added in molar excess as indicated. The positions of the DNA-protein

complexes in (b) and (c) are indicated by arrowhead and at the bottom the free probes are indicated.

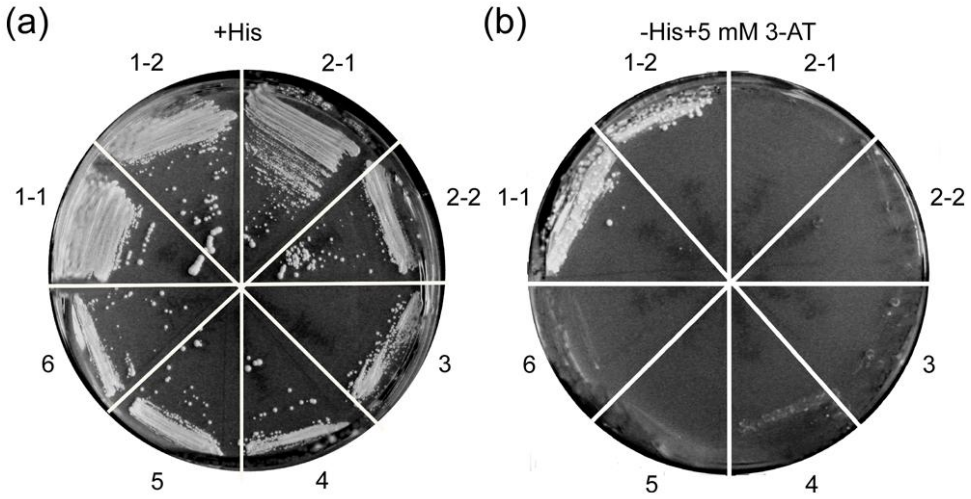

Figure 5. Effects of *OsDof25* overexpression on *OsC4PPDK* promoter-*HIS3* reporter constructs in yeast one-hybrid assays

Reporter constructs ProOsC4PPDK-WT::HIS3, ProOsC4PPDK-MU::HIS3 and an control *HIS3* construct from pINT1-HIS3NB were integrated into the genome of yeast strain Y187 and the resulting strains Y187*:*ProOsC4PPDK-WT, Y187*:*ProOsC4PPDK-MU and YPO101 were transformed with pACTIIa-OsDof25 or pACTIIa as empty vector control. Transformed strains were streaked on medium containing histidine (**a**) or on medium without histidine but with 5 mM 3-AT (**b**). Construct pACTIIa-OsDof25 in Y187:ProOsC4PPDK-WT (sectors 1-1, 1-2), pACTIIa-OsDof25 in Y187:ProOsC4PPDK-MU (sectors 2-1, 2-2), pACTIIa-OsDof25 in YPO101 (sector 3), pACTIIa in Y187: ProOsC4PPDK-WT (sector 4), pACTIIa in Y187:ProOsC4PPDK-MU (sector 5), pACTIIa in YPO101 (sector 6). pACTIIa-OsDof25 shows growth on histidine-lacking medium when grown in a strain with construct ProOsC4PPDK-WT::HIS3, but not with ProOsC4PPDK-MU::HIS3 in which the *OsC4PPDK* promoter is mutated or in the control strain YPO101 that contains a control *HIS3* gene preceded by a minimal TATA box-containing promoter.

Initially, a loss-of-function approach identified a 111 bp fragment in the *OsC4PPDK* promoter which is important for regulation by OsDof25. Further EMSAs and yeast one-hybrid studies confirmed specific binding of OsDof25 with a CTTT sequence in this fragment. In order to further confirm the interaction of OsDof25 protein with this fragment *in planta* another series of GUS reporter plasmids were made which encompassed a series of five constructs (ProPPDK-A::GUS, ProPPDK-B::GUS, ProPPDK-C::GUS, ProPPDK-D::GUS and ProPPDK-E::GUS) for a gain-of-function approach in combination with transient expression in protoplasts. These constructs were based on the 111 bp fragment from -385 to -274 bp and represented the wild type fragment and four mutants in the CTTT motif (Table S2). As shown in Fig. 6, the wild type fragment (construct ProPPDK-A::GUS) was activated by OsDof25 by five-fold, but when either of the four mutant fragments are used, the ratio of induction drops down to two-fold as the empty constructs. Together, the

results show that OsDof25 can interact *in vitro* and *in vivo* specifically with a Dof binding motif close to the ORF of *OsC4PPDK* and may play an important role *in planta* regulation too. Since OsDof24 is the closest homologue in the phylogeny tree with OsDof25 (Fig. 2), we also tested if this gene is able to activate the same *OsC4PPDK* promoter constructs as used in the loss-of-function and gain-of-function analyses. As shown in Fig. S1, the effects of OsDof24 expression in the protoplast system are essentially the same as with OsDof25 (Fig. 3 and 6).

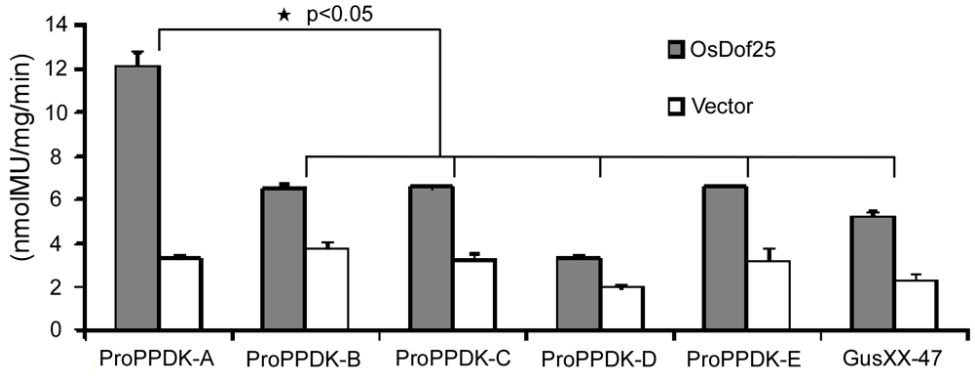

Figure 6. Interaction of OsDof25 with wild type and mutant fragments (-385 to -274) from the *OsC4PPDK* promoter.
Effects of OsDof25 overexpression on regulation of *OsC4PPDK* promoter GUS constructs. The GUS reporter construct ProPPDK-A::GUS bears the wild type fragment (-384 to -274) from the *OsC4PPDK* promoter containing motif CTTT. In constructs ProPPDK-B::GUS, ProPPDK-C::GUS, ProPPDK-D::GUS and ProPPDK-E::GUS the wild type motif CTTT is mutated into GTTT, CATT, CTAT and CTTA, respectively. Plasmid pGusXX-47 was used as a negative control for the reporter. The reporter plasmids were co-transformed with Pro35S::OsDof25, or empty effector pRT100. Relative GUS activities were normalized for total protein. The bar graphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates.

Sub-cellular localisation of OsDof25 transcription factor in rice protoplasts

The subcellular localisation of OsDof25 protein was studied using transient expression of a GFP-tagged OsDof25 fusion protein in rice protoplasts. For this, the ORF of *OsDof25* was fused in frame to the N-terminus of the *GFP* gene to generate plasmid Pro35S::OsDof25-GFP. The empty plasmid pTH2 (Pro35::GFP) was used as a control (Chiu et al. 1996). Fluorescence was detected using Confocal Laser Scanning Microscopy (CSLM). As shown in Fig. 7, GFP-tagged OsDof25 was specifically localised in the cell nucleus, whereas the control GFP protein was localised in both cytoplasm and the nucleus. This experiment confirmed that the OsDof25 protein is a nuclear-localised protein which is consistent with a function as transcription factor.

Expression profiling of *OsDof25* and *OsC4PPDK*

The expression profile of *OsDof25* was studied using qPCR and promoter GUS plants. The qPCR assays showed that the expression of *OsDof25* is not strictly tissue-specific, but has highest expression in penultimate leaves at 10 DAF (days after flowering) and two week old seedlings, followed by flag leaves at 10 DAF, panicle, leaf sheath and stem and lowest expression in roots (Fig 8a). To further study the expression pattern of *OsDof25* and the possible overlap with *OsC4PPDK* in more detail, we generated ProOsDof25::GUS and ProOsC4PPDK::GUS transgenic lines. For both *OsDof25* and *OsC4PPDK* promoters, the GUS signal was observed in leaves and florets as well as in germinating seeds but there was no GUS signal detected in radicles which is in accordance to the qPCR data for *OsDof25*. For *OsC4PPDK*, GUS activity was also detected in immature seeds, where no expression for *OsDof25* could be detected (Fig. 8b).

Taken together, the results demonstrated that *OsDof25* is expressed in different tissues and at different developmental stages. The GUS signals detected in ProOsDof25::GUS rice are in accordance with the expression profiles of *OsDof25* determined by qPCR and overlap with those of *OsC4PPDK*, indicating that OsDof25 may play an important role in rice growth and development besides playing a key role on the transcription of *OsC4PPDK* but we cannot rule out entirely different functions.

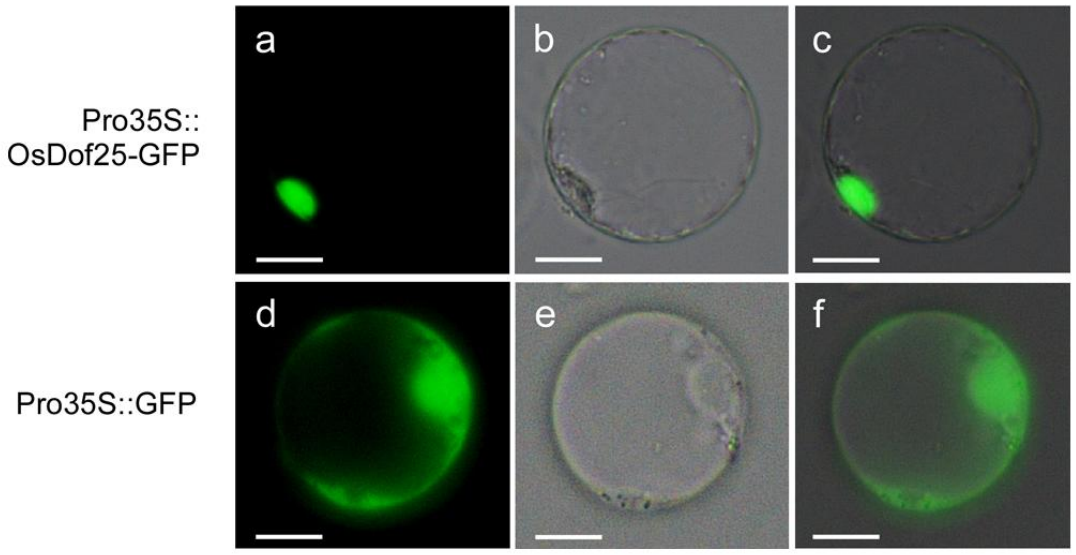

Figure 7. Sub-cellular localisation of OsDof25 protein in rice seedling protoplasts.
Rice protoplasts were transiently transformed with Pro35S::OsDof25-GFP (**a, b, c**) and Pro35S::GFP (**d, e, f**). After overnight incubation, cells were observed with fluorescence (**a, d**) and bright field (**b, e**) microscopy. A merged picture of **a** and **b** and **d** and **e**, respectively, were shown in panels **c** and **f**. Scale bar represent 2 µm.

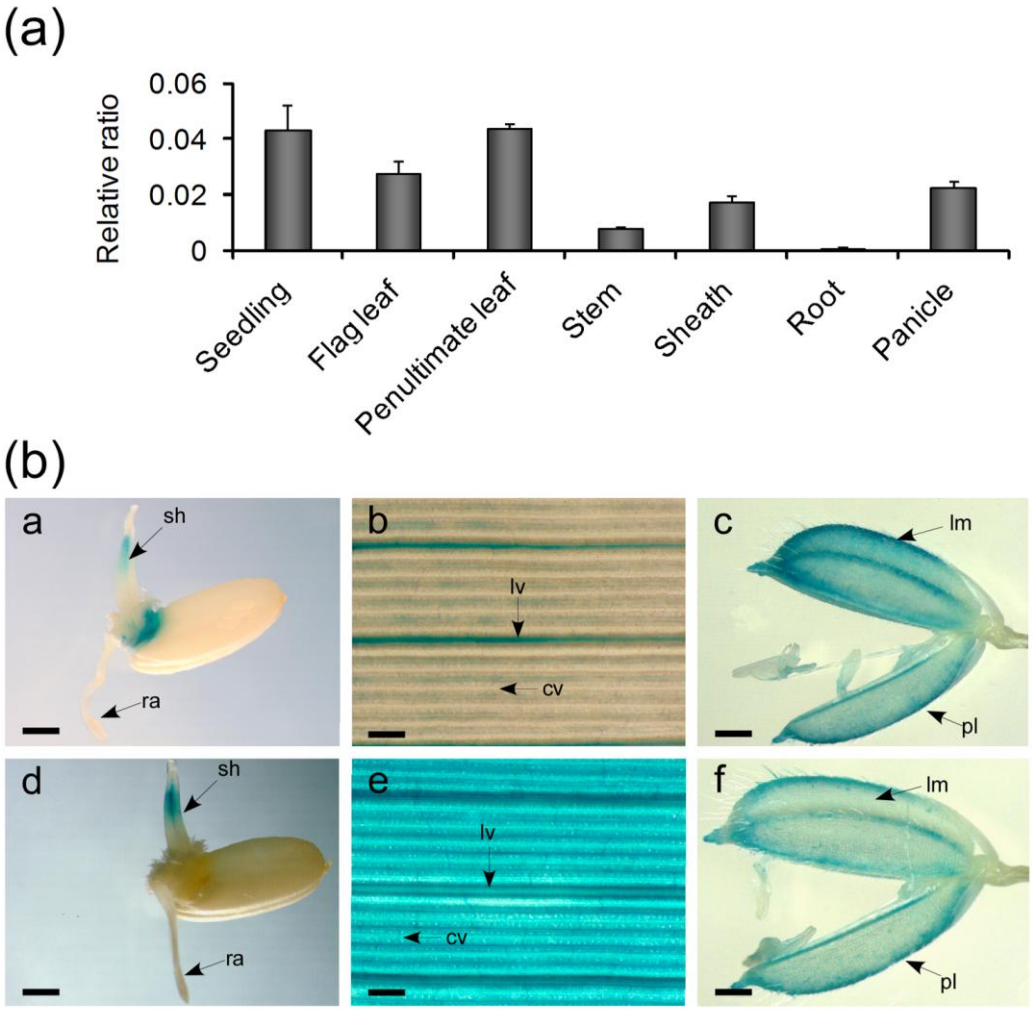

Figure 8. Characterisation of the expression profile of *OsDof25* and *OsC4PPDK.*
(**a**) Expression profile of *OsDof25* in different tissues which are from left to right, two week old seedlings, 10 DAF (days after flowering) flag leaf, penultimate leaf, stem, sheath, panicle and root. The *Ubi* gene was used as control for normalisation of cDNA quantity. Bars represent means standard error (n=3 independent qPCRs).
(**b**) Histochemical localisation of *OsDof25* (**a**-**c**) and *OsC4PPDK* (**d**-**f**) promoter-GUS expression in transgenic rice. (**a**-**d**) Two days old-germinating seeds, (**b**-**e**) Flag leaf, (**c**-**f**) Immature spikelets. Scale bars represent 1 mm (**a, d**), 100 µm (**b, e**) and 500 µm (**c, f**) respectively.
Abbreviations: cv, commissural vein; lv; longitudinal vein; lm, lemma; pl, palea; ra, radicle; sh, shoot.

Functions of OsDof25 in regulation of *OsC4PPDK* expression in rice

The regulation of *OsC4PPDK* by OsDof25 was further studied using loss-of-function and gain-of-function approaches in transgenic rice. To determine whether OsDof25 is required for expression of the *OsC4PPDK* gene, we tried to down-regulate *OsDof25* expression using RNA interference (RNAi) with the pHANNIBAL system (Wesley et al. 2001). For this, a binary vector was made carrying an inverted repeat of a specific part of the *OsDof25* and used in rice transformation. Five independent

single copy transgenic lines were identified using Southern blotting analysis (Fig. S2a) and used in further analysis. Among the five lines transformed with the *OsDof25* silencing construct, two lines, numbers #5 and #30, showed a decrease in *OsDof25* mRNA level as shown by qPCR analysis (Fig. S2b) but no obvious phenotype was visible. Next, the T_1 generation of RNAi-*OsDof25* line #5 and line #30 were analysed in more detail for the effect on *OsC4PPDK* expression. Both RNAi lines showed a reduction in the level of *OsC4PPDK* mRNA compared to the control lines which were non-transgenic lines separated from the T_0 (Fig. S2c).

The RNAi experiments demonstrated that OsDof25 plays a role in the expression of *OsC4PPDK*. To determine whether an elevated level of *OsDof25* expression is sufficient for activating the expression of *OsC4PPDK*, transgenic rice plants were made, which were transformed with a binary vector carrying the *OsDof25* gene driven by the constitutive *GOS2* promoter (De Pater et al. 1992; Ouwerkerk et al. 2001). Three independent single copy number lines were selected using Southern blotting (Fig. S3a) and qPCR assays showed that all three transgenic lines had significantly elevated *OsDof25* mRNA levels compared to the control lines (Fig. S3b). These lines did not show obvious differences in phenotype but analysis of the expression level of *OsC4PPDK* by qPCR showed significant up-regulation (Fig. S3c). Since *OsC4PPDK* is important in photosynthesis we also checked photosynthetic capacity in the *OsDof25* overexpressors and RNAi lines but we did not observe a significant and large difference with the wild type lines (Fig. S4). Taken together, both loss-of-function and gain-of-function studies with OsDof25 showed a clear and opposite effect as activator of *OsC4PPDK* expression which confirmed the results obtained from the EMSA, yeast one-hybrid assay and transient expression experiments in rice protoplasts where we showed binding of OsDof25 with the *OsC4PPDK* promoter.

Discussion

In a comparative study between *Dof* genes from rice and maize we identified the paralogous pair *OsDof24* and *OsDof25* as orthologues of *ZmDof1*. Little is known about the precise functions of Dof genes although some members were characterised in rice (Gaur et al. 2011; Nie et al. 2013), Arabidopsis, Sorghum (Kushwaha et al. 2011), tomato (Cao et al. 2013), *Brachypodium* (Hernando-Amado et al. 2012) and soybean (Guo et al. 2013). In rice for *OsDof12* (Li et al. 2009), a function in flowering time was described in detail. *OsDof24* is also named *OsDof25* because of a different nomenclature (Santos et al. 2012) and has been implicated in regulation of genes involved in carbon and nitrogen metabolism but this is a different gene than the *OsDof25* gene described here. Other *Dof* genes such as *BPBF* from barley (Diaz et al. 2002) and several genes from *Brachypodium* (Hernando-Amado et al. 2012) are expressed during grain development and seem to play a role in this process but this seems not be the case for *OsDof25* showing that although *Dof* genes bind similar *cis*-acting elements, they may still have functions in different

tissues and biological processes. More than half of the Dof members in Arabidopsis are expressed in vascular tissues and may have roles in vascular development and functioning such as short or long-distance signaling (Le Hir and Bellini 2013). We found that *OsDof25* was also expressed in vascular tissue, which suggests that this gene could also play a role in development of vascular tissues or in regulation of genes involved in vascular loading or unloading or other processes acting in this tissue.

In maize, *Dof1* is known to have functions in regulation of *C4PPDK* (Yanagisawa 2000) which plays an important role in C4 photosynthesis (Chollet et al. 1996). Since *OsDof25* is a close homologue of *ZmDof1* which is involved in regulation of *PPDK*, we conducted several studies to check whether this gene-gene interaction is conserved in rice. In order to understand the expression of the mechanism by which OsDof25 potentially could regulate *OsC4PPDK*, we delineated the *OsC4PPDK* promoter and identified a minimal fragment with only one Dof binding site that still gave activation by OsDof25 in a transient expression analysis. Functionality of this site in regulating *OsC4PPDK* expression was proven by mutation analysis that showed reduced binding in *in vitro* and in yeast one-hybrid experiments which was next confirmed with the transient protoplast experiments. Important data for a role of OsDof25 in regulation of *OsC4PPDK* came from a loss-of-function and gain-of-function analysis where down-regulated or up-regulated *OsDof25* expression correlated with lower or higher *OsC4PPDK* expression respectively. Furthermore, no adverse phenotypical effects were seen in these transgenics which also increases the usefulness in biotechnological applications. Because OsDof24 is closest to OsDof25, we also did some analyses on this gene and found that it is also able to regulate the *OsC4PPDK* promoter in rice protoplasts in the same way as OsDof25 did. However, although we did not check for *OsC4PPDK* expression in *OsDof24* overexpression rice transgenics, we came to the conclusion that these genes will have different downstream targets since these plants showed an obvious delay in flowering time and strong effects on flowering time genes were observed (Yu et al., unpublished results).

The overlap in expression patterns of the *OsDof25* and *OsC4PPDK* genes in green tissues is confirmed by the promoter GUS analyses and is in accordance with the transgenic experiments that show that OsDof25 is a key activating regulator of *OsC4PPDK.* Future experiments will have to show whether this is also the case for other photosynthesis genes in either the C3 or C4 pathways. Since *OsDof25* is also expressed in non-photosynthetically active tissues, this activator will likely also be involved in completely other pathways. Although rice is not a typical C4 plant like maize or Sorghum since it does not have the typical Kranz anatomy to capture CO_2 nor does rice have the accompanying photosynthetic capacity, C4 photosynthesis genes are present (Bao et al. 2005; Song et al. 2010). Chinese hybrid rice combinations can show a slightly elevated photosynthetic capacity and it has been speculated that enhanced activity of C4 photosynthesis enzymes is responsible for

this phenomenon (Bao et al. 2005; Song et al. 2010) but it needs still to be investigated if the *Dof* genes such as *OsDof25* are involved in any differential expression of downstream targets. We also observed that the photosynthetic capacity of *OsDof25* overexpression and RNAi lines was not different from the controls. However, transgenic overexpression studies with the maize *PEPC* and *PPDK* genes in rice and wheat showed a clear effect on photosynthetic capacity and certain yield components such as increased grain yield also because of increased tillering (Ku et al. 2001; Zhang et al. 2014). However, these experiments are quite different than our set-up where *OsC4PPDK* was higher expressed due to overexpression of an upstream regulator which may mean that *OsC4PPDK* expression is not higher in all cell types.

To summarise, in this study we confirmed an *in planta* interaction between the *cis*-element of the *OsC4PPDK* promoter and OsDof25 and we confirmed that this gene is an activator of *OsC4PPDK*. However, since photosynthetic capacity is not increased due to *OsDof25* over-expression, likely also other genes will be required to achieve this and increase yield in this way.

Materials and Methods

Sequence alignments and phylogeny analysis

The sequence of the ZmDof1 protein was obtained from GenBank ABF51012 (Yanagisawa 2001; Cavalar et al. 2007). All protein sequences of rice Dof transcription factors were downloaded from the rice transcription factor database (http://ricetfdb.bio.uni-potsdam.de/v2.1/) (Riano-Pachon et al. 2007). The amino acid sequences of ZmDof1 and the rice Dof proteins were aligned using ClustalX (2.0). The Neighbor-Joining algorithm implemented in MEG4 (Tamura et al. 2007) was used for the phylogenetic tree assay. Two hundred bootstrapped data sets were used to estimate the confidence of each clade tree.

RNA isolation, RT-PCR, gene cloning and sequence analysis

Flag leaves harvested 10 DAF (days after flowering) from rice hybrid Liangyou 2186 (*Oryza sativa* L. ssp. *indica*) were ground in liquid nitrogen and total RNA was extracted by using Trizol according to the manufacturer's instructions (Invitrogen). Genomic DNA contaminants were removed from RNA samples by incubating with DNA-free™ (Ambion) at 37°C for 30 minutes. First-strand cDNA was synthesised starting from 1 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) as described by the manufacturer.

The RT-PCR reactions were performed based on single-strand cDNA. The primer OsDof25-F1 and OsDof25-R1 (Table S3) were used to get the cDNA of *OsDof25*. PCR conditions were 5 min of initial denaturation at 98°C, 36 cycles of denaturation at 98°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for

45 s, followed by a final extension step at 72°C for 10 min. The PCR products were separated in a 1x TBE, 1% agarose gel.

To confirm the identity of the amplified sequences, the PCR products were cloned in pCR-Blunt II-TOPO (Invitrogen) and sequenced commercially (BaseClear, Leiden, The Netherlands). The sequences of *OsDof25* were further analysed using DNAMAN.

Construction of effector and reporter plasmids, transient transformation of rice protoplasts and GUS assays

An effector plasmid, Pro35S-OsDof25, was made by insertion of an *Xho*I-*Kpn*I fragment from pCR-blunt II-TOPO-OsDof25 into expression vector pRT100 (Töpfer et al. 1987). For the loss-of-function analysis, GUS reporter plasmids carrying different lengths of the *OsC4PPDK* promoter were generated by insertion of different PCR fragments of the *OsC4PPDK* promoter using the *Bam*HI and *Nco*I sites into vector pGusXX (Pasquali et al. 1994). For the gain-of-function analysis, wild type sequence and mutant fragments from -385 to -274 of *OsC4PPDK* promoter were obtained by PCR and then digested with *Not*I/*Spe*I and inserted into plasmid pGusXX-47 (Pasquali et al. 1994) which has a minimal TATA box containing fragment from the CaMV 35S promoter. Reporter plasmids ProPPDK-A::GUS, ProPPDK-B::GUS, ProPPDK-C::GUS, ProPPDK-D::GUS and ProPPDK-E::GUS (Fig. 6) represent the fragment containing wild type CTTT motif, mutant motifs GTTT, CATT, CTAT and CTTA respectively. The primers used to construct the reporter plasmids construction are listed in Table S3.

Starting material for the protoplast experiments were two weeks old seedlings. Seeds of rice cultivar Minghui 86 (*Oryza sativa* L. ssp. *indica*) were allowed to imbibe in water at darkness at room temperature for three days. Next, the seeds were sown in soil and grown at 26°C, 80% humidity and 12/12 dark/light period, with a light intensity (photosynthetically active radiation value) of 180 µmol/m^2/s. Protoplast isolation and transfection was essentially performed as described by Chen (2006). Cotransformations were performed with 4 µg reporter plasmids and 6 µg effector plasmids using a PEG-based transformation method. Co-transformations with empty overexpression vectors served as controls. After incubation in 1.5 ml W5 buffer in a dark room at 28°C overnight, protoplasts were harvested and total protein were isolated and then frozen in liquid nitrogen. GUS activity assays were performed as described by Van der Fits and Memelink (1997) and protein concentrations were measured using the Bradford protein assay reagent (BioRad). Each experiment was performed at least three times and the relative GUS activities of duplicate samples were normalised for total protein.

Recombinant OsDof25 protein expression and Electrophoretic Mobility Shift Assays (EMSA)

The fragment of *OsDof25* cDNA was fused in-frame with the GST sequence in expression vector pGEX-KG (Guan and Dixon 1991) by sub-cloning *Bam*HI-*Eco*RI fragments from construct Pro35::OsDof25. In order to extract recombinant protein, 5 ml of overnight cultures of BL21 (DE3 pLys) (Novagen) carrying pGEX-KG-OsDof25 plasmids were used to inoculate 500 ml LB medium containing 200 µg/ml carbenicillin and 50 µg/ml chloramphenicol, which was incubated at 37°C to OD600 0.5. Next, protein synthesis was induced by the addition of solid IPTG to final concentration of 1 mM and cultures were incubated for 4 h at 29°C. The harvested cells were suspended in 20 ml PBS and frozen in liquid nitrogen. After thawing pellets at 37°C, the bacteria were lysed by sonication (eight times 10 s burst; 5 s pause between bursts), and centrifuged (at 18,000 rpm for 30 min at 4°C), then the supernatant was filtered through a 0.45 µm membrane. Protein purification was performed using Poly-Prep Chromatography columns (Biorad 731-1550) containing 0.5 ml settled Glutathion-Sepharose 4B beads (Amersham Biosciences). Columns were first washed two times with 10 ml PBS before bacterial extract was passed through. After binding, columns were washing with 10 ml PBS and bound proteins were eluted in 2.5 ml (10 x 0.25 ml) glutathion elution buffer (100 mM glutathione, 500 mM Tris-HCL pH 8.0). Eluted protein was concentrated using Microcone centrifugal filter devices (Millipore) according to manufacturer's instruction and the protein content was determined by the method of Bradford. The GST-OsDof25 fusion protein was stored at -80°C in 10% glycerol.

All EMSA reactions contained 100 ng poly-(dI-dC)-poly-(dI-dC) (Amersham-Pharmacia) and 1 ng of ^{32}P end-labeled probe (~10^8 cpm/µg) in nuclear extraction buffer (Green et al. 1987). Labeled probes were incubated with GST-OsDof25 proteins together at room temperature 30 min and then were loading on a native 4% polyacrylamide (30:0.8) gel in 0.5x TBE while under current. Probes used in labeling originated from annealed oligonucleotides are listed in Table S2.

DNA binding specificity of OsDof25 proteins in yeast one-hybrid assay

Fragments P3 and P6 from the *C4PPDK* promoter, containing the putative Dof binding motif CTTT and its mutant derivative CTAT, respectively, were obtained by annealing the primers listed in Table S2 and then cloned into yeast integrative vector pINT1-HIS3NB (GenBank Accession AY061966; Ouwerkerk and Meijer 2011) between *Not*I and *Spe*I sites. The resulting plasmids ProOsC4PPDK-WT::HIS3 and ProOsC4PPDK-MU::HIS3 were confirmed by sequencing. Next, the *HIS3* reporter-containing fragments were excised with *Sac*I-*Nco*I and introduced into yeast strain Y187 (*MAT*α, *ura3-52*, *his3-*Δ*200*, *ade2-101*, *trp1-901*, *leu2-3, 112*, *met*$^-$, *gal4 gal80*, *URA3:GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ;* Clontech) (Meijer et al. 1998; Ouwerkerk and Meijer 2001, 2011) resulting in yeast strains Y187:ProOsC4PPDK-WT and

Y187:ProOsC4PPDK-MU. YPO101 is a control strain used in yeast one-hybrid assays.

An *Eco*RI/*Bam*HI fragment from pRT100-OsDof25 containing the *OsDof25* ORF was inserted into pACTIIa (Meijer et al. 1997) to generate plasmid pACTIIa-OsDof25, which has a fusion between OsDof25 and the GAL4 AD in order to carry out DNA binding assays in a yeast one-hybrid system. For this, plasmid pACTIIa-OsDof25 was introduced into yeast strains Y187:ProOsC4PPDK-WT, Y187:ProOsC4PPDK-MU and YPO101. The empty plasmid pACTIIa was transformed into the same strains as negative control.

Yeast transformations were performed as earlier described (Ouwerkerk and Meijer 2001). The transformed yeast cells were plated on CM/-Leu-His+Met+Ade+Trp medium and incubated at 30°C and usually after four or five days colonies appeared. Next, the resulting colonies were streaked on CM/-Leu+His+Met+Ade+Trp medium including different concentrations of 3-AT. The plates were incubated at 30°C for one week before scoring. All handlings with yeast were as described earlier (Meijer et al. 2000; Ouwerkerk and Meijer 2001, 2011).

Sub-cellular localisation of OsDof25 protein

The ORF of *OsDof25* was amplified with primers OsDof25-F2 and OsDof25-R2 (Table S3) from the Topo vector. The PCR product was fused in frame to the N-terminus of the green fluorescent protein (GFP) gene to generate plasmid Pro35S::OsDof25::GFP. Vector pTH2 (Pro35S::GFP) was used as a control (Chiu et al. 1996). In this plasmid the S65T sGFP gene is driven by the CaMV 35S promoter and no specific localisation signals are present. Protoplasts isolated from two weeks old rice seedlings were transiently transformed using a PEG-mediated method (Chen et al. 2006). The transformed protoplasts were incubated at 28°C overnight in K3 buffer and then checked for expression using a then observed using a Nikon Eclipse Ci fluorescence microscope. Excitation and emission filters for GFP detection were Ex470-490/DM505/BA520-560.

Binary vector construction and plant transformation

To generate a *OsDof25* (LOC_Os09g29960) promoter GUS fusion construct, a 2,368 bp DNA sequence upstream of the predicted *OsDof25* translation start site was amplified by PCR from genomic DNA isolated from Minghui 86 using Phusion polymerase (Invitrogen) and primers ProOsDof25-F and ProOsDof25-R (Table S3). The PCR fragment was subsequently cloned into vector pCR2.1 Topo (Invitrogen) for sequence analysis and then inserted into vector pCAMBIA-1391Z (GenBank Accession AF234312) resulting in a fusion with the GUS reporter gene. An *OsC4PPDK* promoter GUS fusion construct (ProOsC4PPDK::GUS) was generated by insertion of a 2,572 bp *Eco*RI-*Nco*I fragment *OsC4PPDK* promoter, into pCAMBIA-1391Z. The resulting constructs were used for rice transformation.

To construct an *OsDof25* vector for overexpression, the full length coding sequence was amplified by PCR with primers OsDof25-F5 and OsDof25-R5 (Table S3) and then inserted as *Nco*I/*Bam*HI fragment between the *GOS2* promoter and *nos* terminator in binary vector pCAMBIA-1300intC (GenBank Accession AF294978). The resulting construct was used for rice transformation. The expression of *OsDof25* was down-regulated by an RNAi approach based on generating transgenic plants equipped with a pHANNIBAL silencing vector (Wesley et al. 2001). Specific *OsDof25* sense and anti-sense sequences were obtained by PCR and then inserted into pHANNIBAL in a two-step cloning procedure. First, the anti-sense fragment of *OsDof25* was generated using primers OsDof25-F6 and OsDof25-R6 (Table S3), then digested by *Cla*I/*Bam*HI, and inserted into pHANNIBAL vector between the same sites. Second, the sense fragment of *OsDof25* was generated using primers OsDof25-F7 and OsDof25-R7 (Table S3) and then digested with *Xho*I/*Kpn*I, and inserted into pHANNIBAL already carrying the anti-sense fragment. The resulting plasmid was digested with *Sal*I/*Spe*I and the generating fragments was inserted into pCAMBIA-1300intC between *Sal*I and *Xba*I. The resulting binary vector was used in rice transformation.

The *OsC4PPDK* promoter (LOC_Os05g33570) was obtained by PCR on genomic DNA isolated from indica rice cultivars Minghui 86 and SE21S using primers ProOsC4PPDK-F and ProOsC4PPDK-R (Table S3). To confirm the identity of the amplified sequences, PCR products were cloned in pCR-Blunt II-TOPO vector (Invitrogen) and sequenced by BaseClear (Leiden, The Netherlands).

Transformation of the *japonica* rice cultivar Zhonghua 11 with the above descried binary vector constructs was performed as described by Scarpella (Scarpella et al. 2000) except that *A. tumefaciens* LBA4404 was used for all rice transformations. Prior to growth in the greenhouse, transgenic seedlings were selected on a half-strength Murashige-Skoog medium supplied with 0.7% type I agarose (Sigma) and 25 µg/ml hygromycin. By using Southern blotting with *hpt* as a probe, we identified single copy T-DNA lines.

Detection of GUS expression in transgenic rice, cytological techniques and microscopy

Histochemical detection of GUS activity, cytological techniques and microscopy were performed as described previous (Scarpella et al. 2000). Samples were viewed using a Leica MZ12 stereo microscope or a Leitz Diaplan microscope with bright-field optics settings and images were acquired with a Sony 3CCD Digital Photo Camera DKC-5000.

Expression assays of OsDof25 and OsC4PPDK using qPCR

To study the expression profile of *OsDof25* and *OsC4PPDK*, a qPCR analysis was done on a collection of nine tissues, including two-week old seedlings and eight different tissue samples from mature Minghui 86 plants, including stems, roots,

sheath, flag leaves, penultimate leaves and panicles at 10 DAF (days after flowering). For the expression analysis of *OsDof25* and *OsC4PPDK* in over-expression and RNAi transgenic lines, total RNA was isolated from 10 DAF flag leaves using Trizol kit (Invitrogen). RT reactions were performed with SuperScript™ II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Reactions were performed in an optical 96-well plate with an ABI PRISM® 7500 Real-time PCR System (Applied Biosystems) by using SYBR® Green to monitor dsDNA synthesis. All reactions contained 12.5 µl 2× SYBR® Green Master Mix Reagent (Applied Biosystems), 2.0 ng cDNA and 10 pmol of each gene-specific primer in a final volume of 20 µl. Thermal cycling was as follows: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec. Relative expression levels of reporter and target genes were calculated using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen 2001) using rice *Actin1* and *Ubiquitin* as internal control. An overview of primers used in qPCR is shown in Table S4.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank databases with accession codes KC996732, KC996733 and KP772258 for the *OsC4PPDK* promoter, the *OsDof25* cDNA clone and the *OsDof25* promoter, respectively.

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Author Contributions Statement

MW, ZZ and PBFO designed the project. ZY and PBFO designed the research and wrote the article. ZY and NIV performed experiments and MW and PBFO contributed to analysis and interpretation of results.

Conflict of Interest Disclosure

The authors declare that they have no conflict of interest.

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Supplemental data

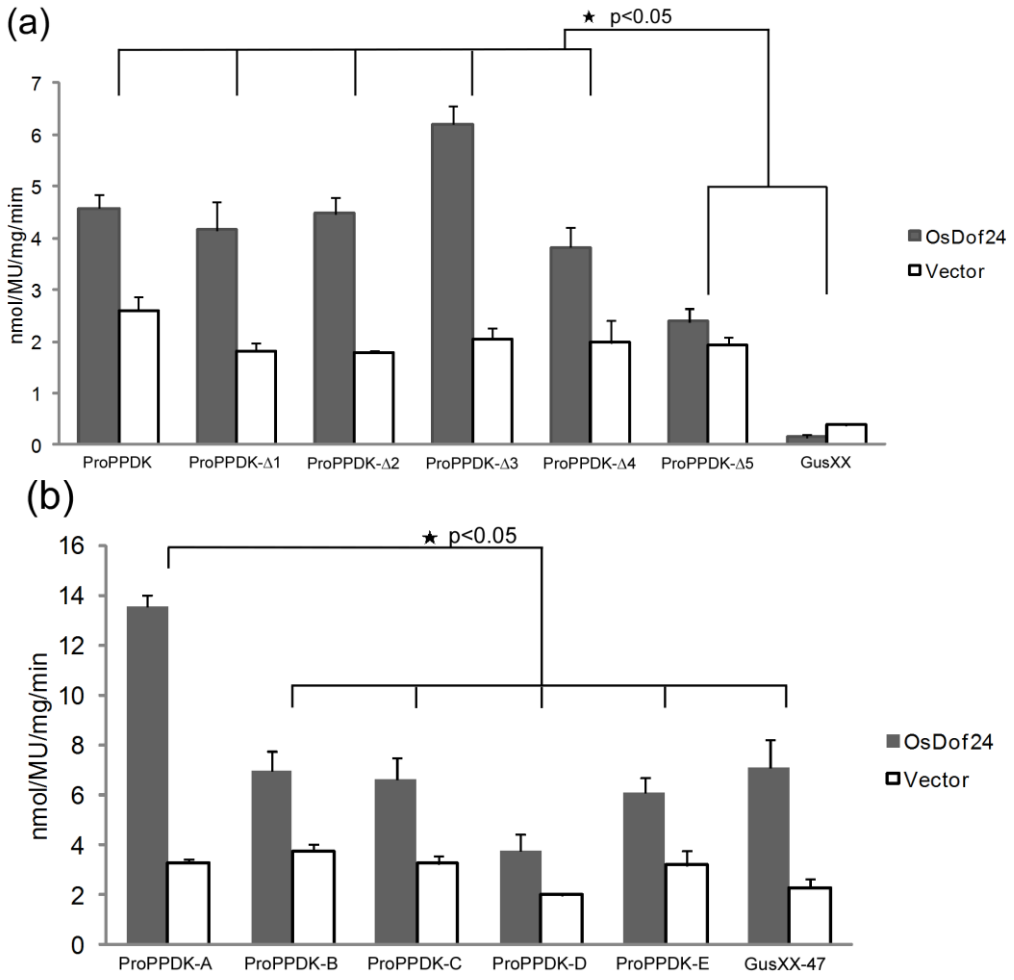

Figure S1. Interactions of OsDof24 with the *OsC4PPDK* promoter in rice protoplasts.
(a) Loss-of-function analysis of the *OsC4PPDK* promoter**.**
Effects of OsDof24 overexpression and the mapping of OsDof24-binding fragments on the *OsC4PPDK* promoter were tested using an overexpression construct Pro35S::OsDof24 which was co-transformed into rice protoplasts with a series of *OsC4PPDK* promoter deletion GUS constructs. GUS activities of co-transformation with Pro35S::OsDof24 are indicated in black and columns representing empty effector plasmids are blank.
(**b**) Gain-of-function analysis of OsDof25 with wild type and mutant fragments (-385 to -274) from the *OsC4PPDK* promoter.
The GUS reporter construct ProPPDK-A::GUS bears the wild type fragment (-384 to -274) from the *OsC4PPDK* promoter containing motif CTTT. In constructs ProPPDK-B::GUS, ProPPDK-C::GUS, ProPPDK-D::GUS and ProPPDK-E::GUS the wild type motif CTTT is mutated into GTTT, CATT, CTAT and CTTA, respectively. Plasmid pGusXX-47 was used as a negative control for the reporter. In both panels, the reporter plasmids were co-transformed with Pro35S::OsDof24, or empty effector pRT100. Relative GUS activities were normalised for total protein. The bar graphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates. The data were analysed using ANOVA followed by

Bonferroni corrections. Asterisks indicate significant differences (p<0.05) compared with the untransformed controls.

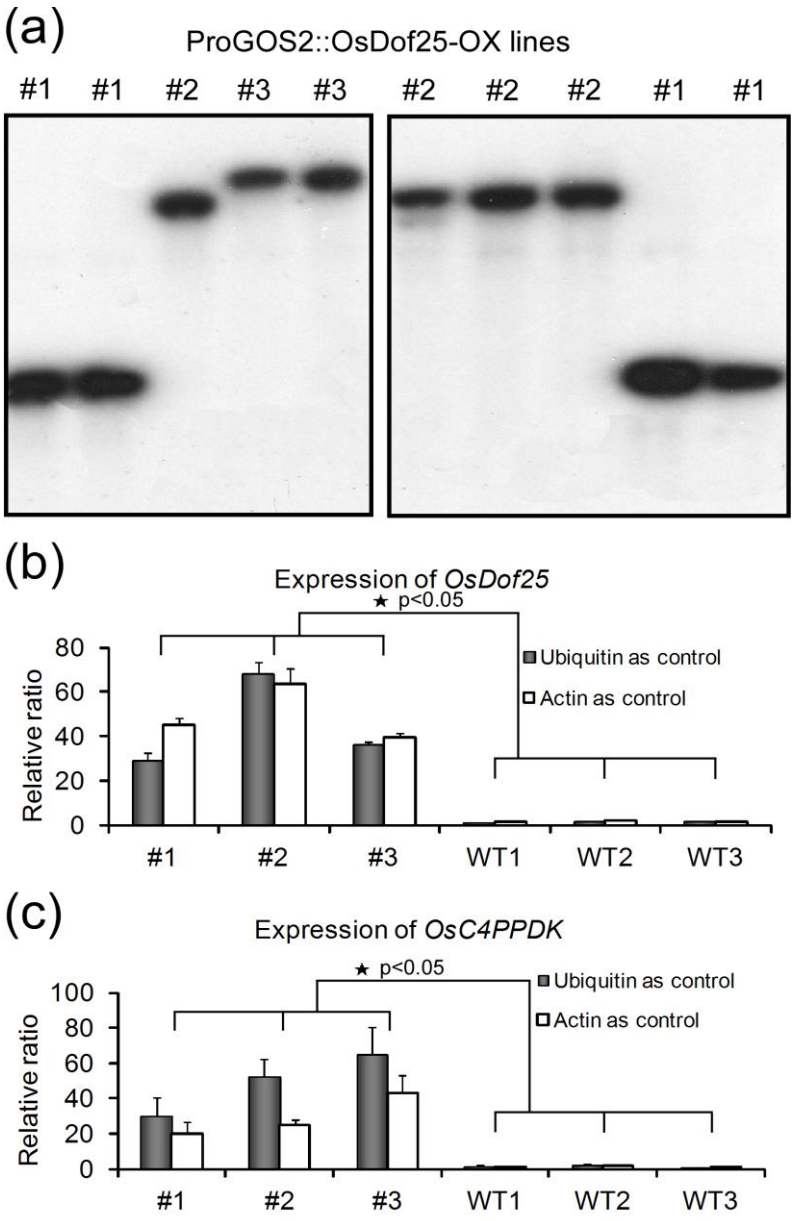

Figure S2. Molecular analysis of *OsDof25* RNAi plants.
(**a**) Southern blotting results showing the T-DNA insertion copy number. The *hpt* gene was used as probe. (**b**) Analysis of *OsDof25* expression in T_1 *OsDof25* RNAi plants (lines #5 and #30) and control plants, azygous plants separated from the T_0 (WT). (**c**) Analysis of *OsC4PPDK* expression in T_1 *OsDof25* RNAi plants (lines #5 and #30) and control plants, which are azygous plants separated from the T_0 (WT). *Ubiquitin* and *Actin* genes were used for equilibration of cDNA quantity in qPCR experiments. Bars represent means standard error (n=3 independent qPCRs). The bargraphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SE) of biological replicates. The data were analysed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences (p<0.05) compared with the untransformed controls.

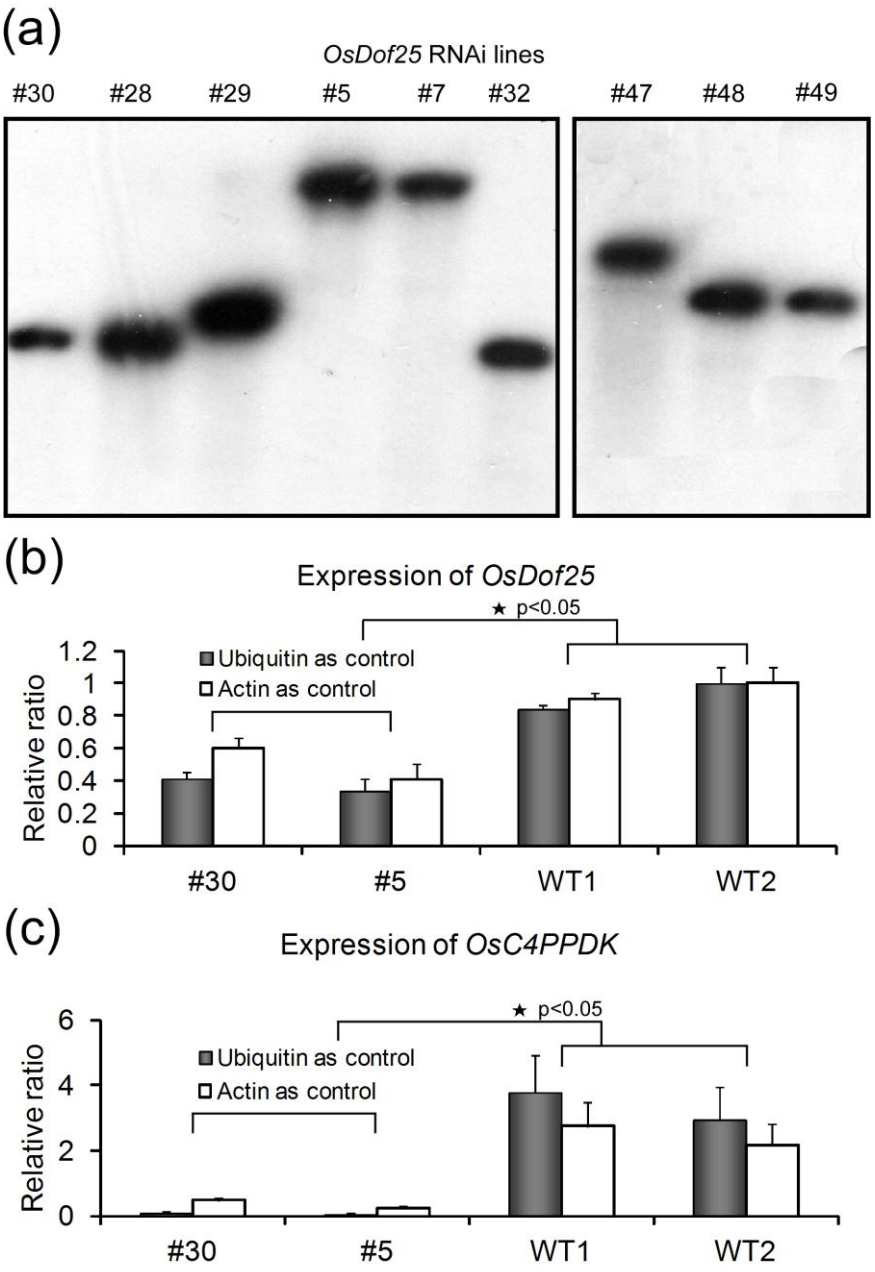

Figure S3. Molecular analysis of transgenic plants overexpressing *OsDof25.*
(**a**) Southern blotting results showing the T-DNA insertion copy number. The *hpt* gene was used as probe. (**b**) Expression analysis of *OsDof25* in T_1 ProGOS2::OsDof25 plants (lines #1, #2 and #3) and azygous controls segregated from the T_0 (WT). (**c**) Expression analysis of *OsC4PPDK* in ProGOS2::OsDof25 T_1 plants (lines #1, #2 and #3) and azygous control plants, segregated from the T_0 (WT). *Ubiquitin* and *Actin* genes were used for equilibration of cDNA quantity in qPCR experiments. The bar graphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates. The data were analysed using ANOVA followed by Bonferroni corrections. Asterisks indicate significant differences ($p<0.05$) compared with the untransformed controls.

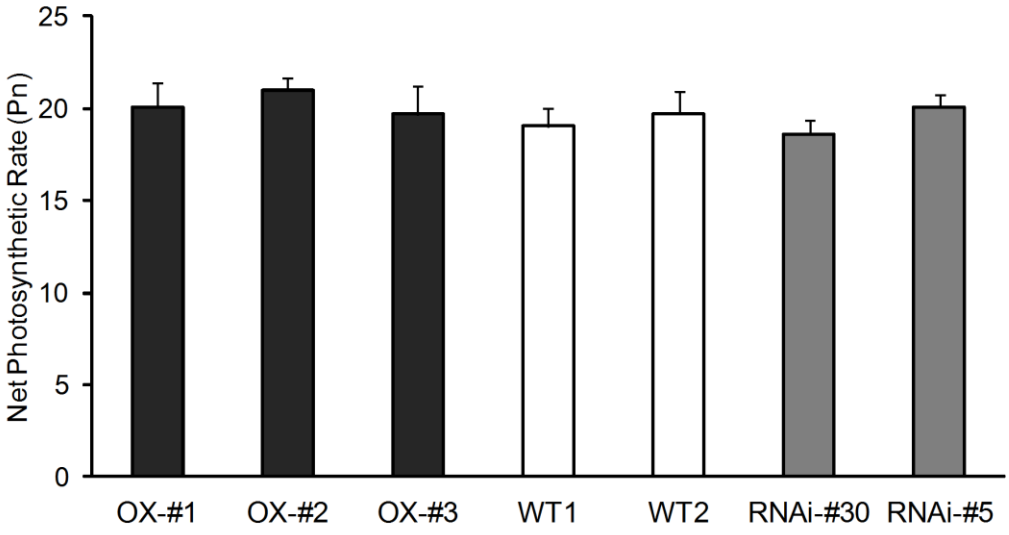

Figure S4. Analysis of the photosynthetic capacity in OsDof25-OX and RNAi lines. Photosynthetic capacity was determined under field conditions at day 5 after flowering on flag leaves from OsDof25 overexpression and RNAi plants and compared to control plants. The bar graphs are based on the mean values of three independent plants of each line and error bars represent the standard deviation (SD) of biological replicates.

Table S1. Overview of the Dof transcription factor family as identified in japonica rice cultivar Nipponbare (Lijavetzky et al. 2003; Yang et al. 2006; Jin et al. 2014). Indicated are the gene names, locus codes in TIGR, chromosomal coordinates in Mb and GenBank Accession codes for BAC or PAC clones.

Gene code	Japonica BAC/PAC clone	Position on chromosome (cm)	Locus code	Chromosome number
OsDof1	P0505D12	37.8	LOC_Os01g64590	1
OsDof2	P0453A06	8.9	LOC_Os01g15900	1
OsDof3	P0671B11	5	LOC_Os01g09720	1
OsDof4	P0038F12	9.7	LOC_Os01g17000	1
OsDof5	P0007F06	28	LOC_Os01g48290	1
OsDof6	B1131G08	32.1	LOC_Os01g55340	1
OsDof7	P0680A05	29.2	LOC_Os02g47810	2
OsDof8	B1121A12	30.2	LOC_Os02g49440	2
OsDof9	P0657H12	27.4	LOC_Os02g45200	2
OsDof10	OSJNBa0009N02	8.6	LOC_Os02g15350	2
OsDof11	OSJNBa0010D22	21.5	LOC_Os03g38870	3
OsDof12	OSJNBa0091P11	3.7	LOC_Os03g07360	3
OsDof13	OSJNBa0063J18	23.4	LOC_Os03g42200	3
OsDof14	OSJNBb0014A21	9.3	LOC_Os03g16850	3
OsDof15	OSJNBa0079B15	31.6	LOC_Os03g55610	3
OsDof16	OJ1754_E06	34.4	LOC_Os03g60630	3
OsDof17	OSJNBa0064G10	34.4	LOC_Os04g58190	4
OsDof18	OSJNB0005N02	28.3	LOC_Os04g47990	4
OsDof19	P0016H04	0.6	LOC_Os05g02150	5
OsDof20	P0491D10	10.1	LOC_Os06g17410	6
OsDof21	P0407H12	7.6	LOC_Os07g13260	7
OsDof22	OJ1163_G04	19.3	LOC_Os07g32510	7
OsDof23	OSJNBa0060O17	29	LOC_Os07g48570	7
OsDof24	P0605H02	24.1	LOC_Os08g38220	8
OsDof25	P0556A05	18.2	LOC_Os09g29960	9
OsDof26	OSJNBa0060A14	13.5	LOC_Os10g26620	10
OsDof27	OSJNBa0066I08	18.5	LOC_Os10g35300	10
OsDof28	OSJNBa0016C14	23.4	LOC_Os12g38200	12
OsDof29	B1110B01	21.4	LOC_Os05g36900	5
OsDof30	OSJNBa0044E20	24.7	LOC_Os12g39990	12

Table S2. List of oligonucleotides used in EMSA and yeast experiments. Overview of sequences of both wild type and mutant fragments of the *OsC4PPDK* promoter, which were used in EMSA and yeast experiments. The putative Dof protein binding motifs, CTTT, are labeled in bold and the mutant series are indicated by underlines.

Name	Sequence (5' to 3')
P1 up	GGCCTTGTAAATACACTAAAATTACATATGTAAT
P1 down	CTAGATTACATATGTAATTTTAGTGTATTTACAA
P2up	GGCCTTAGTGACTTACAATGTAAATACATGCC
P2 down	CTAGGGCATGTATTTACATTGTAAGTCACTAA
P3up	GGCCCCGACTAA**CTTT**TGATGAAAAATATG
P3 down	CTAGCATATTTTTCATCAAAAGTTAGTCGG
P4 up	GGCCCCGACTAA**G**__**TTT**__TGATGAAAAATATG
P4 down	CTAGCATATTTTTCATCAAAACTTAGTCGG
P5 up	GGCCCCGACTAAC__**A**__**TT**TGATGAAAAATATG
P5down	CTAGCATATTTTTCATCAAATGTTAGTCGG
P6 up	GGCCCCGACTAA**CT**__**A**__**T**TGATGAAAAATATG
P6 down	CTAGCATATTTTTCATCAATAGTTAGTCGG
P7 up	GGCCCCGACTAA**CTT**__**A**__TGATGAAAAATATG
P7 down	CTAGCATATTTTTCATCATAAGTTAGTCGG

Table S3. List of oligonucleotides used for the construction of GUS reporter plasmids, and used in PCR. Putative Dof binding motifs in the *OsC4PPDK* promoter are indicated in bold and mutant sites are indicated by underlining.

Name	Sequences (from 5' to 3')
ProPPDK-F	ATAAGAATGCGGCCGCGTACTTACATATATAATTTTG
ProPPDK-R-A	GGACTAGTCCATATTTTTCATCA**AAAG**TTAG
ProPPDK-R-B	GGACTAGTCCATATTTTTCATCA**AAAC**TTAG
ProPPDK-R-C	GGACTAGTCCATATTTTTCATCA**AATG**TTAG
ProPPDK-R-D	GGACTAGTCCATATTTTTCATCA**ATAG**TTAG
ProPPDK-R-E	GGACTAGTCCATATTTTTCATCA**TAAG**TTAG
ProOsC4PPDK-F	GGAATTCCTGGTTTCCACGGCGTGCAACGGTGGC
ProOsC4PPDK-R	CCATGGCCTGGCCCTGATCGATC
OsDof25-F1	CGGGATCCATGCAGGAGGCGGGGCGAC
OsDof25-R1	GGAATTCTCCATGGCAGGTTAAGGAACAGTG
OsDof25-F2	CCGCTCGAGATGCAGGAGGCGGGGCGAC
OsDof25-R2	CATGCCATGGCTGGCAGGTTAAGGAACAG
OsDof25-F3	TAATACGACTCACTATAGGGCACAACCAAGAAAGCCCCGG
OsDof25-R3	TGGCAGGTTAAGGAACAGTGATGG
OsDof25-F4	TAATACGACTCACTATAGGGTGGCAGGTTAAGGAACAGTGATGG
OsDof25-R4	CACAACCAAGAAAGCCCCGG
OsDof25-F5	CATGCCATGGAGGAGGCGGGGCGACGG
OsDof25-R5	CGGGATCCTCATGGCAGGTTAAGGAAC
OsDof25-F6	CCATCGATATCGGTGTCGGCGAACAC
OsDof25-R6	CGGGATCCCACCACCGCCGACGCTG
OsDof25-F7	CCGCTCGAGCACCACCGCCGACGCTG
OsDof25-R7	GGGGTACCATCGGTGTCGGCGAACAC
ProOsDof25-F	CCTGAGAACTCAGACGCTATGACGAAAC
ProOsDof25-R	CCATGGCGCGCCCGCAGAAAAGCTCGGACGAAC

Table S4. List of oligonucleotides used for qPCR

Name	Sequences (from 5' to 3')
*Actin*F	GACCCAGATCATGTTTGAGACC
*Actin*R	CATCACCAGAGTCCAACACAATAC
*Ubiquitin*F	AGCAGAAGCACAAGCACAAG
*Ubiquitin*R	AAGCCTGCTGGTTGTAGACG
OsDof25-F	AAGGAGCACCTGGACACGA
OsDof25-R	GGCAGGTTAAGGAACAGTGATGGA
OsC4PPDK-F	GATCCGTCGTGGCGCAGA
OsC4PPDK-R	CTGAGAGGCGAGCAATGC

Chapter 4

OsDof24 and *OsDof25* activated the promoter of *GluB-1* and influenced the rice seeds protein

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Key words (listed in order of importance): Dof, *GluB-1*, rice, seed storage protein

Abstract

OsDof24 and OsDof25 belong to the so-called Dof class of transcription factors, which contain one highly conserved one zinc finger DNA-binding domain, hence the name DNA binding with One Finger. Cotransfection of OsDof24 and OsDof25 overexpression constructs with a *GluB-1* core-promoter construct resulted in activation of *GUS* expression. We present evidence that OsDof24 and OsDof25 can *trans*-activate the *GluB-1* promoter by binding to two putative Dof-binding motifs within a 25 bp region in the *GluB-1* promoter. First, the OsDof25 protein specifically recognized the Dof binding motif in the *GluB-1* promoter as shown by EMSA (Elecrophoretic Mobility Shift Assays). Secondly, cotransfection of OsDof24 and OsDof25 with the *GluB-1* promoter mutated in a Dof binding motif suppressed the transactivation capacity of both OsDof24 and OsDof25. Furthermore, nitrogen concentrations of grains from OsDof24 or OsDof25 overexpressing and RNAi transgenic lines were different. Taken together these data indicated that rice OsDof24 and OsDof25 have functions in controlling *GluB-1* expression levels.

Introduction

Seed storage proteins are specifically synthesized during seed maturation and have functions in the structure of the grain but are also important nitrogen and carbon resources for the germinating seedling (Chen *et al*. 2012). Apart from that, seed storage proteins are the primary source of protein in human diets and animal feed worldwide. Seed storage proteins can be grouped into albumins, globulins, prolamins and glutelins according to their solubility properties in solvents. Most cereal species, such as maize and wheat accumulate prolamins as their major seed storage protein, but rice is unique among cereal species as it accumulates significant amounts (65%-85%) of glutelin as the major storage protein. Glutelin genes are classified into four subfamilies which are *GluA*, *GluB*, *GluC* and *GluD* (Okita *et al*. 1989; Takaiwa *et al*. 1991; Kawakatsu *et al*. 2008; Qu et al. 2008).

The spatial and temporal specific expression of storage protein genes is primarily controlled by *cis*-regulatory elements which are recognized specifically by transcriptional factors which in many cases are specifically

expressed during grain development (Zheng *et al*. 1993; Wu *et al.* 2000). Several motifs, such as the GCN4 box, the prolamin box (P box), AACA and ACGT motifs, have been identified as *cis*-regulatory elements involved in the endosperm specific expression of cereal seed storage proteins (Zheng *et al*. 1993; Takaiwa *et al*. 1996; Washida et al. 1999; Wu et al. 2000). The rice bZIP transcription activator RITA-1 is highly expressed during seed development and is specifically binding to ACGT motifs (Izawa *et al*. 1994). The GCN4 motif is recognized by basic leucine zipper (bZIP) activators, such as maize Opaque2 (O2) (Vicente-Carbajosa *et al*. 1997; Wu *et al*. 1998), barley BLZ2 (Vicente-Carbajosa *et al*. 1998), wheat SPA1 (Albani *et al*. 1997) and rice RISBZ1 (Onodera *et al.* 2001). The P-box is bound by Dof (DNA binding with one finger) transcription factors including maize PBF (Wang *et al*. 1998; Marzabal *et al*. 2008), barley BPBF (Mena *et al*. 1998; Diaz et al. 2002; Mena et al. 2002), wheat WPBF (Dong *et al.* 2007) and rice RPBF (also named *OsDof3,* (Yamamoto *et al.* 2006; Kawakatsu *et al.* 2009). In rice the combinatorial interactions between RISBZ1 and RPBF play an important role in the regulation of genes expressed in seed (Chen *et al.* 2006; Yamamoto *et al*. 2006; Kawakatsu *et al*. 2009).

Dof proteins are members of a plant specific transcription factor family sharing a highly conserved DNA-binding domain (Yanagisawa 1996, 2004; Shigyo *et al.* 2007; Negi *et al.* 2013). The name of Dof coined from DNA-binding with one single C2-C2 zinc finger (Umemura et al. 2004). The Dof domain is known to be a bi-functional domain which participates in not only DNA-binding but also protein-protein interactions (Zhang *et al*. 1995). The high conservation of the Dof domain in different Dof proteins suggests that they display similar DNA-binding specificity. Dof proteins recognize a conserved AAAG motif or its reversibly orientated sequence, CTTT. The only exception is a pumpkin Dof protein (AOBP) that recognizes an AGTA motif (Yanagisawa 2002). The sequences outside the Dof domain are highly variable suggesting that the functions of Dof proteins might be diverse and there is accumulating evidence that this is indeed the fact. Since the first Dof protein was identified in maize (Yanagisawa and Izui 1993), numerous Dof proteins have been reported having roles in diverse plant-specific processes such as response to plant hormones including gibberellin (Mena *et al*. 2002), auxin (Kisu *et al*. 1997, 1998; Baumann *et al*. 1999) and salicylic acid (Kang and Singh 2000; Kang *et*

al. 2003) and development including seed germination (Dong *et a*l. 2007; Moreno-Risueno *et al*. 2007); stomatal guard cell opening (Plesch *et al*. 2001; Cominelli *et al.* 2011; Negi *et al.* 2013); light responses (Yanagisawa and Sheen 1998; Yanagisawa 2000); the control of grain size (Iwamoto et al. 2009) as well as vascular development (Diaz *et al.* 2002; Le Hir and Bellini 2013).

In the whole rice genome 30 genes for putative Dof transcription factors have been identified (Lijavetzky *et al*. 2003; Riano-Pachon *et al.* 2007). Transcription factor RPBF (*OsDof3*) was reported to interact with RISBZ1 and together regulate seed storage protein genes. *OsDof12* was reported to regulate heading date (Li *et al*. 2009). In our previous studies we have reported that *OsDof24* and *OsDof25* are involved in regulation of the heading date and the *OsC4PPDK* promoter, respectively. In this study, we analyzed the role of OsDof24 and OsDof25 in the regulation of the *GluB-1* promoter. Based on *in vitro* and *in vivo* studies, we propose that OsDof24 and OsDof25 may be involved in the regulation of seed storage protein expression which is supported by the results obtained from overexpression and RNAi transgenic lines.

Results

OsDof24 and OsDof25 are activators of the *GluB-1* promoter

Protoplasts isolated from rice seedlings were transfected to examine the effect of OsDof24 and OsDof25 as effectors on the expression of a chimeric GUS construct driven by the rice glutelin *GluB-1* core promoter (Figure 1). Cotransfections of the *GluB-1-GUS* reporter plasmid with effector plasmids *35S-OsDof24* and *35S-OsDof25*, resulted in an increase of four and three-fold transcriptional activity, respectively, compared with the activity from co-transfection of the pGusXX-47 reporter plasmid and the empty effector plasmid. Analysis of the sequence of the 245 bp *GluB-1* core-promoter revealed a total of seven putative Dof binding motifs with the core sequence AAAG or CTTT. To further investigate the functional relevance and the putative interactions of OsDof24 and OsDof25 with the *GluB-1* promoter, a 25 bp fragment from -144 to -119, with two Dof motifs, was fused with the GUS gene and used in transient expression experiments. Similar results were obtained as when the -245 to +1 fragment of the *GluB-1* promoter was used (Figure 1). These results suggested that both OsDof24 and OsDof25 can

function as activators of the *GluB-1* promoter by interacting with the 25 bp fragment located between -144 and -119.

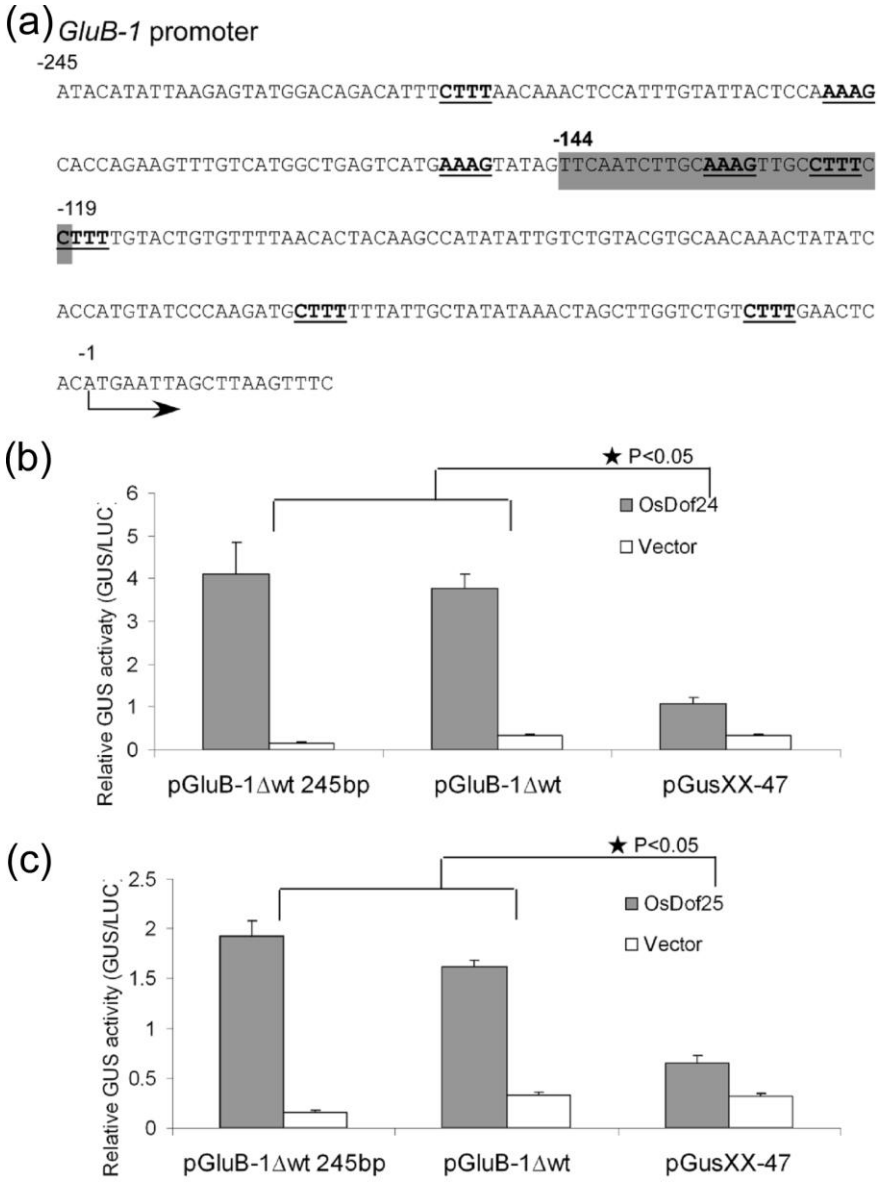

Figure 1. Activation of the *GluB-1* promoter by OsDof24 and OsDof25.

(a) Sequence of the 0.245 kb *GluB-1* promoter. Numerals indicate the nucleotide position from the transcriptional start site, the putative Dof binding motif are underlined and bolded. The 25 bp oligonucleotide region, from -144 to -119, used in the GUS analysis and EMSAs, is shaded in gray.

(b) Rice seedling protoplasts were transiently co-transformed with a *GUS* reporter gene carrying by the *GluB-1* promoter and OsDof24 effector plasmids or empty vector plasmid. The relative GUS activity is shown after normalization of the transformation efficiency. The bargraphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard

deviation (SD) of biological replicates. In each line the means of triplicate transformations was corrected by FLUC (firefly luciferase). Significant differences were indicated by stars (P value <0.05).

(c) Rice seedling protoplasts were transiently co-transformed with a *GUS* reporter gene carrying the *GluB-1* promoter and OsDof25 effector plasmids or empty vector plasmid. The relative GUS activity is shown after normalization the transformation efficiency. The bargraphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates. In each line the means of triplicate transformations was corrected by FLUC (firefly luciferase). Significant differences were indicated by stars (P value <0.05).

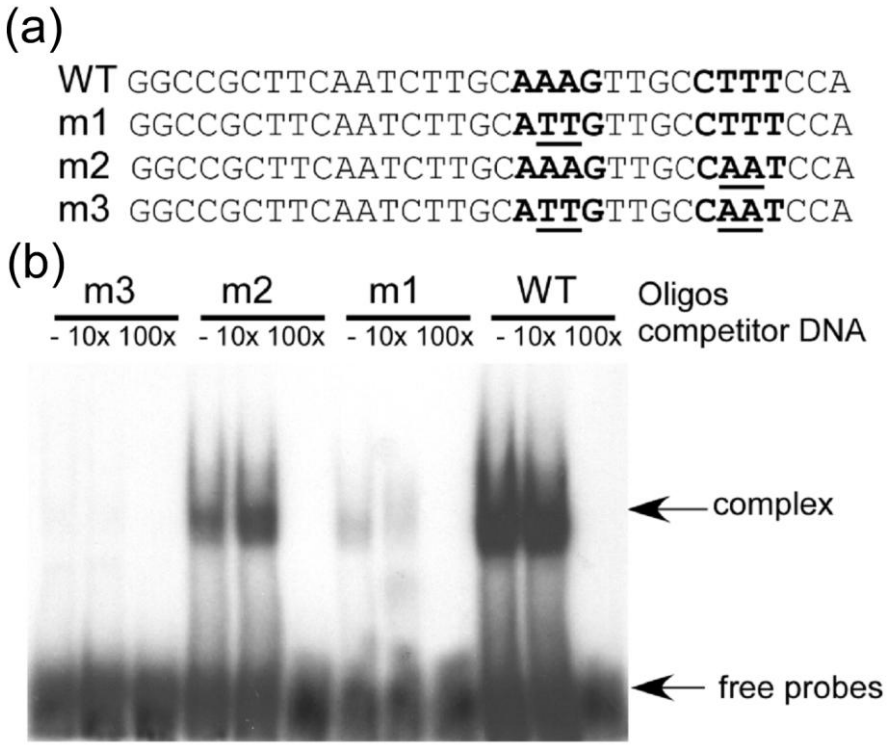

Figure 2. OsDof25 binds *in vitro* to the putative Dof binding motif within the *GluB-1* promoter.

(a) Sequence of part of the *GluB-1* core-promoter. The putative Dof binding motifs, AAAG and CTTT, are in bold, and the mutated nucleotides are underlined. In m1, the first Dof binding site is mutated, in m2 the second Dof binding site is mutated, in m3, both Dof binding sites were mutated.

(b) Electrophoretic Mobility Shift Assay (EMSA) of recombinant OsDof25 protein with the *GluB-1* promoter. Wild type or mutated fragments indicated at the top were used as probes.

In vitro interactions between the *GluB-1* promoter and OsDof25

EMSAs (Electrophoretic Mobility Shift Assays) were used to determine whether OsDof25 can bind directly to the putative Dof binding motif in the *GluB-1* promoter (-144 to -119). These studies could not be repeated with OsDof24 protein because of difficulties with its purification. As shown in Figure 2, retarded protein bands were observed after incubation with OsDof25 with a wild type probe. A 100-fold molar excess of unlabeled competitor abolished this complex, indicating that the *GluB-1* promoter is specifically recognized by OsDof25. To further test the specific recognition of the Dof motif in this fragment by OsDof25, interactions between OsDof25 and 25 bp oligonucleotides containing two putative Dof binding motif within the *GluB-1* promoter (wild type), or the altered putative Dof binding motif (mutant) were used. EMSA analysis of OsDof25 and the 25 bp oligonucleotides containing one mutated and one wild type putative Dof binding motif still give a complex, which also can be abolished by adding 100-fold molar excess of unlabeled competitor. By contrast, use of the 25 bp oligonucleotides containing two mutated putative Dof binding motifs abolished complex formation in the EMSA, indicating that OsDof25 specifically recognized both Dof binding motifs, in this fragment.

Both OsDof24 and OsDof25 activate the promoter of *GluB-1* by interacting with Dof binding motifs

To further study the interactions of OsDof24 and OsDof25 with the *GluB-1* promoter, a series of three mutated GluB-1 promoter fragments based on the -144 to -119 sequence containing mutated Dof binding motif were developed. In m1, AAAG is mutated into ATTG, in m2 CTTT is mutated into CAAT, while m3 has both ATTG and CAAT mutations. As shown in Figure 3, cotransfection of OsDof24 and OsDof25 with the wild type *GluB-1* promoter (-144 to -119) resulted in an increase of 5.5- and 3-fold transcriptional activity respectively compared with the control. Cotransfection of OsDof24 and OsDof25 with mutation m1 (ATTG), resulted in an increase of 3- and 1.5-fold transcriptional activity, respectively. Cotransfection with the *GluB-1* promoter containing mutation m2 (CAAT), resulted in a similar increase as cotransfected with the *GluB-1* promoter containing m1. However, when the *GluB-1* promoter

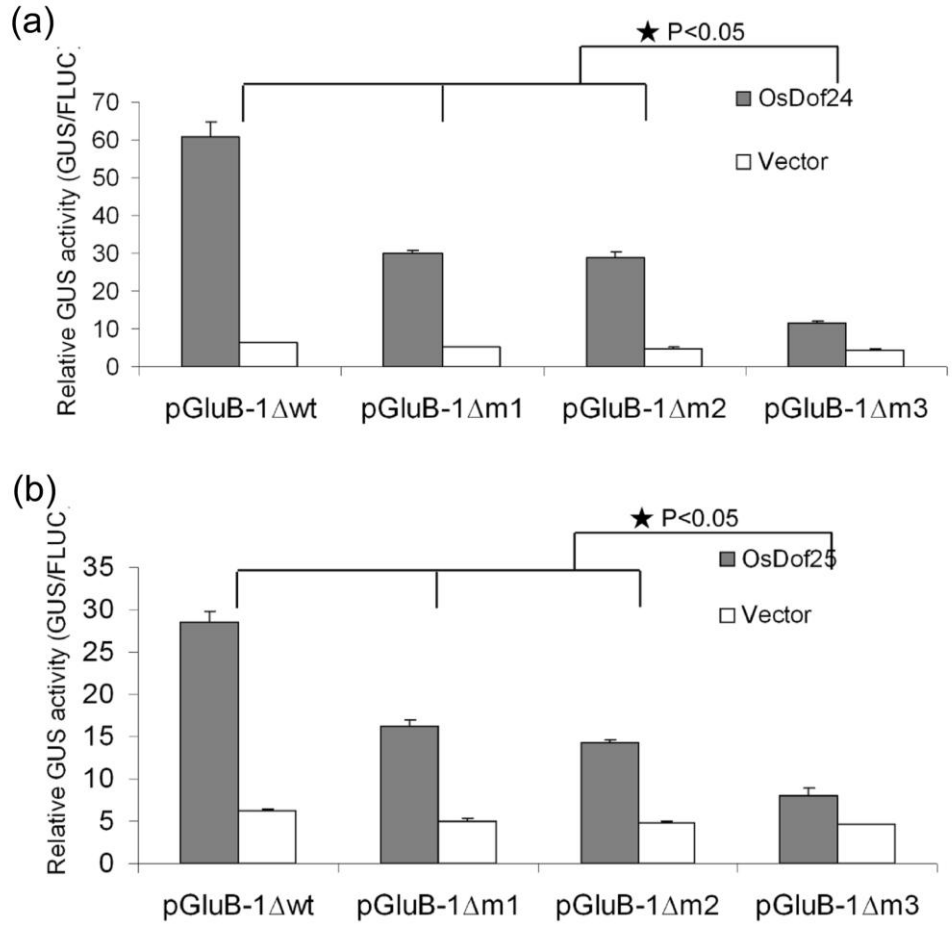

Figure 3. OsDof24 and OsDof25 bind *in vivo* to the putative Dof binding motif within the *GluB-1* promoter

(a) Rice seedling protoplasts were transiently co-transformed with a *GUS* reporter gene driven by variants of the *GluB-1* promoter and OsDof24 effector plasmids or an empty vector plasmid. Reporter plasmid pGluB-1∆wt contains the wild-type *GluB-1* promoter fused with *GUS* reporter gene, while pGluB-1∆m1, pGluB-1∆m2 and pGluB-1m3 have the first, the second and both the first and second Dof binding motif mutated, respectively. The relative GUS activity is shown after normalization for the transformation efficiency. The bargraphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates. In each line the means of triplicate transformations was corrected by FLUC (firefly luciferase). Significant differences were indicated by stars (P value <0.05).

(b) Rice seedling protoplasts were transiently co-transformed with a *GUS* reporter gene driven by variants of the *GluB-1* promoter and OsDof25 effector plasmids or an empty vector plasmid. Reporter plasmid pGluB-1∆wt contains the wild-type *GluB-1* promoter fused with *GUS* reporter gene, while pGluB-1∆m1, pGluB-1∆m2 and

pGluB-1m3 have the first, the second and both the first and second Dof binding motif mutated, respectively. The relative GUS activity is shown after normalization for the transformation efficiency. The bargraphs are based on the mean values of three independent transformations of each construct combination and error bars represent the standard deviation (SD) of biological replicates. In each line the means of triplicate transformations was corrected by FLUC (firefly luciferase). Significant differences were indicated by stars (P value <0.05).

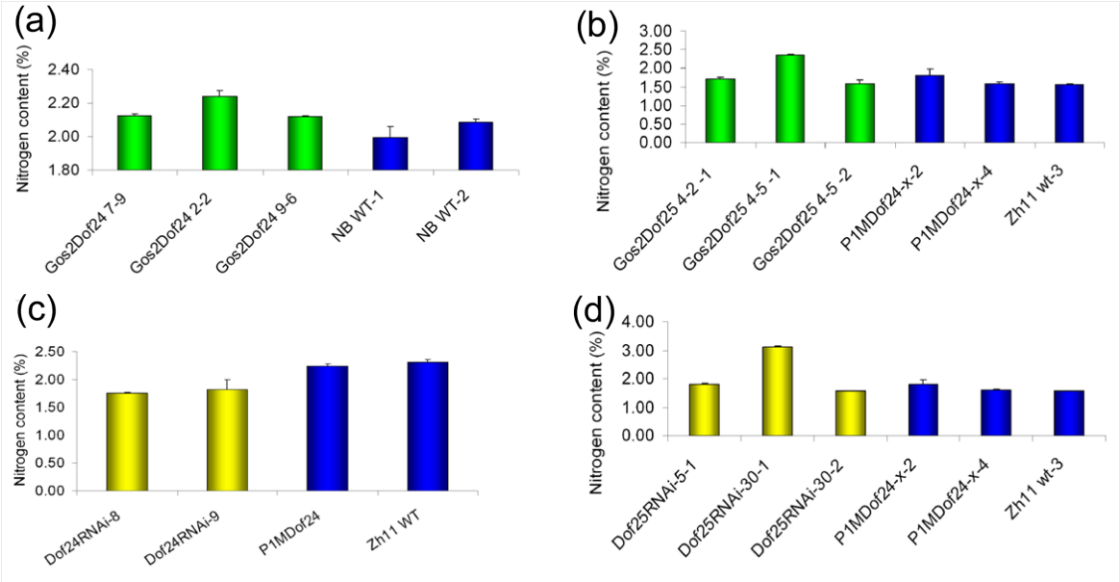

Figure 4. Nitrogen concentration of overexpression and RNAi transgenic seeds compared with the controls.

(a) Comparison of the nitrogen content of the OsDof24 overexpression transgenic seeds and the controls.

(b) Comparison of the nitrogen content of the OsDof25 overexpression transgenic seeds and the controls.

(c) Comparison of the nitrogen content of the OsDof24 RNAi transgenic seeds and the controls.

(d) Comparison of the nitrogen content of the OsDof25 RNAi transgenic seeds and the controls.

Five random seeds from each plant were ground into fine powder. Per sample, 4 mg of powder was used and the nitrogen concentration was measured by dry combustion on a Flash EA 2000 elemental analyzer.

harboring both mutations was used in cotransfections with OsDof24 or OsDof25, activation of expression was much lower than with mutants m1 and m2. Taken together, the results show that in transient expression experiments

using rice protoplasts, both OsDof24 and OsDof25 were able to interact and activate the *GluB-1* promoter, but that they would need both intact Dof binding sites for optimal activation.

Effects of OsDof24 and OsDof25 misexpression on protein content of seeds

To examine the effect of OsDof24 and OsDof25 on the concentration of nitrogen in the seeds, we analyzed both overexpression and RNAi transgenic seeds. The transgenic seeds overexpressing *OsDof24* and *OsDof25* showed a somewhat (1.05 and 1.13 fold, respectively higher nitrogen concentration compared with the controls (Figure 4). The *OsDof24* RNAi transgenic seeds showed a 0.78 fold lower nitrogen content, and the *OsDof25* RNAi transgenic seeds showed the 0.94 times lower nitrogen content except the Dof25 RNAi line 30-1.

Discussion

Seed storage protein genes are specifically and highly expressed in developing grains and their promoters have been used to express a number of transgenes in rice seeds (Wu *et al*. 1998; Qu *et al.* 2004, 2008). In rice glutelin is the dominant seed storage protein and is accounting for 60%-80% of rice seed protein content. This protein is synthesized as a 57 kDa precursor and then processed into 37 kDa acidic and 20 kDa basic subunits. An extensive analysis of the complete rice genome sequences indicated that there are more than 11 glutelin genes in the whole rice genome (Katsube-Tanaka *et al*. 2004). For one of these genes, *GluB-1*, it has been reported that the seed-specific expression is driven by a 245 bp core-promoter in which a GCN4 box is an important functional motif (Wu *et al*. 1998, 2000). RISBZ1 and RPBF are transcription factors that regulate the transcription of *GluB-1* and the endosperm specific expression of the rice seed storage protein genes through the GCN4 and P-box motifs (Onodera *et al*. 2001; Kawakatsu *et al*. 2009). In this chapter, we described that the OsDof24 and OsDof25 transcription factors also have roles as activators of the *GluB-1* promoter and influence the accumulation of rice seed proteins in transgenic rice.

A total of 30 Dof transcription factors have been identified in rice (Yanagisawa 2002; Lijavetzky *et al*. 2003; Gao *et al*. 2006; Riano-Pachon *et al*.

2007). In a phylogenetic analysis, *OsDof24* and *OsDof25* are clustering in the same clade and occur in duplicated chromosome regions (Chapter 2). Despite the sequence homology and phylogenetic relationship, the genes seem to have acquired different functions in evolution since *OsDof24* seems to be involved in regulation of flowering time (Chapter 2), while misexpression of *OsDof25* does not show any effect on flowering time but seems to control the expression of the photosynthesis gene *C4 PPDK* (Chapter 3). In transient expression assays using a protoplast system, we found that both OsDof24 and OsDof25 can activate the 245 bp (-245 to -1) *GluB-1* promoter through the Dof binding motif AAAG or CTTT. Analysis of the *GluB-1* promoter showed that a 25 bp (-144 to -119) region can be bound by OsDof24 or OsDof25 and used as a transcription activating sequence. Binding specificity was further confirmed by EMSA experiments using OsDof25. Nucleotide substitutions in either of the two Dof binding motifs did not fully abolish Dof protein binding, but when both Dof binding sites were mutated, hardly any protein complex was visible. In transient assays, the GUS activity driven by the mutant promoters was lower compared with wild type, but still somewhat higher compared with the empty GUS vector control. Nucleotide substitutions in the two Dof binding motif not only abolished binding in EMSAs, but also eliminated *trans*-activation by OsDof24 and OsDof25 in transient expression assays. Taken together, these experimental results indicate that both OsDof24 and OsDof25 can act as transcriptional activator of the *GluB-1* promoter by interaction with the two Dof binding motifs, AAAG and CTTT.

Transcription factors may form useful tools for engineering the expression of group of target genes. OsDof24 and OsDof25 seem to hold this promise, since they are able activate the transcription of the *GluB-1* promoter and *s*tudies on nitrogen concentrations of *OsDof24* and *OsDof25* overexpression and RNAi transgenic rice lines showed that the nitrogen concentrations were increased in both OsDof24 and OsDof25 overexpressing transgenic seeds, and decreased in RNAi transgenic seeds. In Arabidopsis it was shown that *OsDof24* overexpression altered carbon and nitrogen metabolism under high N-supply (Santos *et al.* 2012; in this article the gene was called OsDof25 due to the use of a different nomenclature) supporting a putative role for this gene in N metabolism in rice grain. Although more experiments need to be done to verify *GluB-1* mRNA and protein levels in the *OsDof24* and *OsDof25*

misexpression lines, first results are promising. These studies may help to improve the capacity of seed storage proteins and might be a tool to improve the quality of rice grain.

Materials and methods

Plasmid constructs

For the construction of the GUS reporter plasmid carrying the promoter of *GluB-1* (-245 to -1), a *GluB-1* sequence (-245 to -1) was PCR-amplified using forward primer, 5'-GCGGCCGCTTAAAGTTAGACTCATCTTCTCAAGCATAAGAG-3', and reverse primer, 5'-ACTAGTCAATAAAAAAGCATCTTGGGATACATGG-3', containing overhanging *Not*I and *Xba*I recognition sites respectively. PCR fragments were digested with *Not*I and *Xba*I and introduced between the corresponding sites of vector pGusXX-47 containing a minimal CaMV 35S promoter coupled to a GUS reporter (Pasquali *et al*. 1994).The wild-type and mutated *GluB-1* promoter (-144 to -119) were generated by annealing the 25 complementary oligonucleotide pairs, from Table 1. These double-stranded oligonucleotides were inserted into the *Not*I and *Spe*I recognition sites of vector pGusXX-47. 35S::OsDof24 and 35S::OsDof24 plasmids were previously described in Chapters 2 and 3.

Transient expression assays

Seeds of rice cultivar Minghui 86 (*Oryza sativa* L. ssp. *indica*) were immersed under water in dark at room temperature for three days. Seeds were then grown in soil with cycles of 12 h light at 26°C, 80% humidity, and of 12 h dark at 20°C, 60% relative humidity, and at a light intensity (photosynthetically active radiation value) of -180 µmol/m2/s. Two weeks old seedlings were used for isolating protoplasts and subsequent transformation following the procedure described by Chen *et al*. (2006). For analysis of the interaction of OsDof24 and OsDof25 with the promoter of *GluB-1*, rice protoplasts were cotransformed in triplicate with 4 µg of reporter plasmid carrying a *GluB-1* promoter-GUS construct, 5 µg of effector plasmids as indicated in the figure legends and 1 µg FLUC (firefly luciferase) reference plasmid. Cotransformations with the empty overexpression vectors served as controls. After transformation 1.5 mL W5 buffer was added to dilute PEG and the

protoplasts were incubated overnight in a dark room at 28°C. Cells were harvested after overnight incubation, and total protein was isolated and frozen in liquid nitrogen. GUS activity assays performed as described by Van der Fits and Memelink (1997) were corrected for FLUC activities and expressed as relative activities compared with the vector control. The data were analyzed using ANOVA followed by Bonferroni corrections.

Expression of recombinant OsDof proteins and Electrophoretic Mobility Shift Assays (EMSA)

Firstly, plasmid pGEX-KG-OsDof25 was transformed into *E. coli* BL21 (DE3, pLys; Novagen). In order to extract recombinant protein, 5 ml of overnight cultures of BL21 carrying the pGEX-KG-OsDof25 plasmid were used to inoculate 500 ml LB medium containing 200 µg/ml carbenicillin and 50 µg/ml chloramphenicol at 37°C to an optical density at OD600 of 0.5. Next, protein synthesis was induced by the addition of solid IPTG to a final concentration of 1 mM and cultures were grown for 4 h at 29°C. The harvested cells were suspended in 20 ml PBS and frozen in liquid nitrogen. After thawing the pellet at 37°C, the bacteria were lysed by sonication (eight times 10 s burst; 5 s pause between bursts), centrifuged (at 18,000 rpm for 30 min at 4°C) and the supernatant was filtered through a 0.45 µm membrane. Protein purification was performed using Poly-Prep Chromatography columns (Biorad 731-1550) containing 0.5 ml settled Glutathion-sepharose 4B beads (Amersham Biosciences). Columns were first washed two times with 10 ml PBS before the bacterial extract was passed through. After binding, columns were washed with 10 ml PBS and bound proteins were eluted in 2.5 ml (10 x 0.25 ml) glutathion elution buffer (100 mM glutathione, 500 mM Tris-HCl pH 8.0). Eluted protein was concentrated using Microcone centrifugal filter devices (Millipore) according to the manufacturer's protocol and the protein content was determined by the method of Bradford. The GST-OsDof25 fusion protein was stored at -80°C in 10% glycerol.

The four oligonucleotide probes (including wild type and mutated sequences) described in Fig. 2, were generated by annealing a complementary single-stranded oligonucleotide that created a four base (GGCC) overhang at the 5'-end. These probes were end labeled with [α32P]-dCTP by filling-in reaction with Klenow polymerase. For each probe,

10-fold and 100-fold molar unlabeled wild type probes were used as competitor DNA. All EMSA reactions contained 100 ng poly-(dI-dC)-poly-(dI-dC) (Amersham-Pharmacia) and 1 ng of ^{32}P end-labeled probe (~10^8 cpm/µg) in 1 x nuclear extraction buffer (Green *et al*. 1987). Labeled probes were incubated with GST-OsDof25 proteins at room temperature during 30 min and then were loaded on a running 4% PAGE (30:0.8) gel in 0.5 fold TBE and sterile water was used instead of protein as negative control for each reaction.

Measurement of nitrogen concentrations in rice seeds

Five random mature seeds from each plant were ground into fine powder. Per sample, 4 mg of powder was weighed in a 5×8 mm tin capsule and the nitrogen concentration was measured by dry combustion on a Flash EA 2000 elemental analyzer (Thermo Electron Corporation, Rodana, Italy). The assays were performed with two replicates and the nitrogen concentrations were analyzed statically by a Student's t-Test.

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Tables

Table 1. Synthetic oligonucleotides used in EMSA and transient assays. Top and bottom strand sequences of both wild type and mutated fragments are shown. The putative Dof protein binding motifs are labeled in bold and the mutants are shown by underlined letters.

Name	Sequence (5' to 3')
wt F	GGCCGCTTCAATCTTGC**AAAG**TTGC**CTTT**CCA
wt R	CTAGTGGAAAGGCAACTTTGCAAGATTGAAGC
m1 F	GGCCGCTTCAATCTTGC**ATTG**TTGC**CTTT**CCA
m1 R	CTAGTGGAAAGGCAACAATGCAAGATTGAAGC
m2 F	GGCCGCTTCAATCTTGC**AAAG**TTGC**CAAT**CCA
m2 R	CTAGTGGATTGGCAACTTTGCAAGATTGAAGC
m3 F	GGCCGCTTCAATCTTGC**ATTG**TTGC**CAAT**CCA
m3 R	CTAGTGGATTGGCAACAATGCAAGATTGAAGC

Table 2. Plasmids used in the transient analysis. Plasmids used in the rice protoplasts transient analysis. In each transformation the reporter plasmid, the effector plasmid and the reference plasmid are listed in this table.

Transformation number	Reporter (4 µg)	Effector (5 µg)	Reference (3 µg)
1	pGluB-1Δwt 245bp	OsDof24	FLUC
2	pGluB-1Δwt 245bp	OsDof25	FLUC
3	pGluB-1Δwt 245bp	pRT100	FLUC
4	pGluB-1Δwt	OsDof24	FLUC
5	pGluB-1Δwt	OsDof25	FLUC
6	pGluB-1Δwt	pRT100	FLUC
7	pGluB-1Δm1	OsDof24	FLUC
8	pGluB-1Δm1	OsDof25	FLUC
9	pGluB-1Δm1	pRT100	FLUC
10	pGluB-1Δm2	OsDof24	FLUC
11	pGluB-1Δm2	OsDof25	FLUC
12	pGluB-1Δm2	pRT100	FLUC
13	pGluB-1Δm3	OsDof24	FLUC
14	pGluB-1Δm3	OsDof25	FLUC
15	pGluB-1Δm3	pRT100	FLUC
16	pGusXX-47	OsDof24	FLUC
17	pGusXX-47	OsDof25	FLUC
18	pGusXX-47	pRT100	FLUC

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Chapter 5

Summary

Y. Zhang

Rice (*Oryza sativa*) is an important staple food which is consumed daily by a large part of mankind, especially in Asia. With the increasing demand for rice and the restriction of producing rice on less land per capita, yield is a very important trait to breed for. The yield of rice has been enhanced significantly during the Green Revolution in the sixties of the 20th century, which not only resulted in new cultivars but also of new management and the use of fertilizers and pesticides. The advent of the genomics era with the availability of obtaining whole genome sequences and knowledge of all rice genes will make it possible to speed-up breeding. From genome analysis it has become clear that about 5% of the genes are transcription factors (TFs) that function in binding to DNA thereby regulating expression of other genes. Some of these TFs have important roles in traits and domestication such as opaque2 and TB1 in maize. Other examples are qSH1 which is a BEL1-type homeobox TF regulating seed shattering, and MOC1 controlling tillering in rice.

In this thesis we aimed to gain novel knowledge on functions of Dof TFs by *in vivo* and *in vitro* experiments and to determine if they have functions in agronomical traits. We used the yeast one-hybrid system to study the interaction between Dof proteins and putative *cis*-elements in gene promoters. EMSAs and transient expression experiments using rice protoplasts were used as an *in vivo* method to further validate such interactions. Overexpression and RNAi transgenic rice were generated to help analyzing the functions of OsDof24 and OsDof25 *in planta*.

Chapter 1 summarizes the knowledge so far on TFs that significantly affect agronomic traits in rice. TFs play important roles in gene transcription and regulation. Yet the function of most of the TFs is still unknown and needs to be further investigated. Since Dof genes play important roles in regulation of photosynthesis genes in maize and wheat and other important developmental processes in plants, these genes were chosen as research topic for this thesis,. In **Chapter 2,** the function of *OsDof24* was studied for possible effects on flowering time. The expression profile of *OsDof24* was analyzed by qPCR and in promoter-*GUS* plants, which showed that the expression of *OsDof24* is not tissue specific. For further functional analysis of OsDof24, we generated transgenic *OsDof24*-overexpressing plants, which showed a delay of flowering time under a 12h light/12h dark condition. To investigate how OsDof24 regulated the flowering time in overexpression plants, the expression of flowering time-related genes was investigated by RT-PCR and qPCR. *OsMADS1*, *OsMADS14*, *Hd3a* and *RFT1*, were shown to be downregulated, whereas *OsMADS50* was upregulated, and the expression profiles of *Hd1*, *OsMADS15*, *OsMADS18*, *OsMADS51* and *Ehd1* were unaffected. Based on these results, we hypothesize that *OsDof24* may be involved in determining flowering time by downregulating the expression of

several downstream flowering time genes, especially *Hd3a* and *RFT1* which are two key genes in the flowering time pathway.

OsDof25 is a gene homologous to maize *Dof1* which is known to be regulating expression of the C4 photosynthesis genes PPDK and PEPC. In **Chapter 3**, the role of *OsDof25* in the regulation of the *OsC4PPDK* gene was studied in detail in order to find out whether this interaction also exists in rice. Rice, as a typical C3 plant, has a lower photosynthetic capacity and CO_2 utilization efficiency than C4 plants such as maize and sorghum. C4PPDK is a key enzyme involved in the C4 pathway and is responsible for catalyzing the pyruvate into phosphoenolpyruvate. However, TFs and other regulatory components that regulate expression of this gene in rice are unknown. We found that the expression of *OsDof25* enhanced transcription of *OsC4PPDK* in a transient expression system based on rice seedling protoplasts. By analyzing the promoter of *OsC4PPDK*, one of the putative binding motifs (CTTT), located from -385 to -274 in the promoter of *OsC4PPDK*, was shown to be important for expression and a target site of OsDof25. This was confirmed by both *in vitro* EMSA and yeast one-hybrid studies. A sub-cellular localization study using GFP-tagging showed that OsDof25 is a nuclear localized protein, which is in accordance to its function as a nuclear TF. Finally, over-expression and down-regulation by RNAi of *OsDof25* confirmed its role in activation of the *OsC4PPDK* promoter. This finding suggests an important role for OsDof25 in carbon skeleton metabolism, operating as a transcriptional regulator of key C4 pathway genes.

In **Chapter 4**, a possible role of OsDof24 and OsDof25 in regulating the expression of the rice *GluB-1* gene was studied using a transient expression system. By co-transfection of *OsDof24* and *OsDof25* overexpression constructs in combination with a 245 bp *GluB-1* core-promoter and a truncated *GluB-1* promoter (25 bp from -144 to -119), respectively, we present evidence that OsDof24 and OsDof25 can *trans*-activate the *GluB-1* promoter by binding to two putative Dof-binding motifs within a 25 bp core region in the *GluB-1* promoter. Furthermore, *in vitro* EMSA (Elecrophoretic Mobility Shift Assays) confirmed that the OsDof25 protein specifically bound this Dof binding motif in the *GluB-1* promoter. Finally, the nitrogen content of grains from OsDof24 or OsDof25 over-expressing and RNAi transgenic lines were different compared to the controls. The results in this chapter indicated that rice OsDof24 and OsDof25 play a role in the regulation of the *GluB-1* promoter and that they influence nitrogen storage via seed storage proteins.

In summary, the work in this thesis has led to the characterization and a better understanding of the functions of two Dof transcription factors which we show have functions in controlling flowering time, the regulation of the photosynthesis gene C4 PPDK and seed storage protein expression in rice.

Samenvatting

Rijst (Oryza sativa) is een belangrijk basisvoedsel dat dagelijks geconsumeerd wordt door een groot deel van de wereldbevolking, vooral in Azië. Vanwege de toenemende vraag naar rijst als gevolg van de groei van de wereldbevolking is opbrengst een zeer belangrijke eigenschap om voor te veredelen. De opbrengst van rijst is aanzienlijk verbeterd tijdens de Groene Revolutie in de jaren zestig van de 20e eeuw. Dit leidde niet alleen tot nieuwe, betere cultivars, maar resulteerde ook in toepassing van nieuwe teeltpraktijken en het gebruik van meststoffen en bestrijdingsmiddelen. Het beschikbaar komen van de volledige genoomsequenties van rijst en kennis van alle rijst genen in dit genomics-tijdperk zal naar verwachting gaan bijdragen aan een versnelling van het veredelingsproces. Uit de analyse van het rijstgenoom is gebleken dat ongeveer 5% van de rijstgenen coderen voor transcriptiefactoren (TFn), die aan DNA binden en de expressie van andere genen reguleren. Sommige TFn hebben een belangrijke rol gespeeld bij de domesticatie zoals opaque2 en TB1 in maïs. Andere voorbeelden zijn qSH1, een BEL1-type homeobox TF, dat een rol speelt bij de verspreiding van het zaad en MOC1, dat de vorming van zijscheuten controleert.

In dit proefschrift heb ik geprobeerd nieuwe kennis te genereren over de functies van Dof TFn door in vivo en in vitro experimenten en heb ik met name gekeken of ze agronomisch belangrijke eigenschappen controleren. Ik heb het gist één-hybride systeem gebruikt om de interactie tussen Dof_eiwitten en potentiele cis-elementen in de promotoren van genen te bestuderen. EMSA's (Electrophoretic Mobility Shift Assays) en transiënte expressie_experimenten met rijstprotoplasten werden gedaan om dergelijke interacties verder in vivo te valideren. Overexpressie en RNAi_transgene rijstlijnen werden gegenereerd om de functies van OsDof24 en OsDof25 *in planta* te analyseren.

Hoofdstuk 1 geeft een overzicht van wat tot nu toe bekend is over de relatie tussen TFn en de agronomische eigenschappen van rijst. TFn spelen een belangrijke rol in de regulatie van de transcriptie van genen, maar de specifieke functie van de meeste TFn is nog onbekend en moet verder worden onderzocht. Aangezien Dof-TFn een belangrijke rol spelen bij de regulatie van fotosynthesegenen in maïs en tarwe en ook bij andere belangrijke ontwikkelingsprocessen in planten, werden deze TFn gekozen als onderwerp van onderzoek voor dit proefschrift.

In **hoofdstuk 2** werd onderzocht in welke mate OsDof24 invloed had op de bloeitijd. Het expressieprofiel van OsDof24 werd geanalyseerd door qPCR en in promotor-GUS-planten, waaruit bleek dat de expressie van OsDof24 niet weefselspecifiek is. Voor verdere functionele analyse van OsDof24 genereerde ik transgene OsDof24-overexpressieplanten, die een

vertraagde bloeitijd toonden onder een 12 uur licht / 12 uur donker regime. Om te onderzoeken hoe OsDof24 de bloeitijd beinvloedde in de overexpressieplanten, werd de expressie van bloeitijd-gerelateerde genen onderzocht in deze overexpressieplanten middels RT-PCR en qPCR. De genen coderend voor OsMADS1, OsMADS14, Hd3a en RFT1 bleken te worden down-gereguleerd, terwijl dat coderend voor OsMADS50 werd up-gereguleerd, terwijl de expressieprofielen van de genen coderend voor HD1, OsMADS15, OsMADS18, OsMADS51 en Ehd1 niet veranderd waren. Op basis van deze resultaten, postuleren we dat OsDof24 de bloeitijd beinvloedt door het down-reguleren van de expressie van verscheidene downstream-bloeitijdgenen, met name de sleutelgenen coderend voor Hd3a en RFT1.

OsDof25 is homoloog aan maïs Dof1, waarvan bekend is dat het de expressie van de C4-fotosynthesegenen voor PPDK en PEPC reguleert. Om na te gaan of deze interactie ook bestaat in rijst, werd in **hoofdstuk 3** de rol van OsDof25 in de regulatie van het*OsC4PPDK*-gen in detail bestudeerd. Rijst is een typische C3-plant met een lagere fotosynthesecapaciteit en CO2 gebruiksefficiency dan C4-planten zoals maïs en sorghum. C4PPDK is een sleutelenzym van de C4-route en is verantwoordelijk voor de omzetting van pyruvaat in fosfoenolpyruvaat. De TFn en andere regulerende elementen die de expressie van *OsC4PPDK* reguleren in rijst zijn nog onbekend. We vonden dat de expressie van *OsDof25* leidde tot een verhoogde transcriptie van *OsC4PPDK* in een transiënt expressiesysteem van protoplasten verkregen uit rijstzaailingen. Analyse van de promotor van *OsC4PPDK* leerde dat een van de potentiele bindingsmotieven (CTTT), gelegen op positie -385 tot -274 in de promotor van *OsC4PPDK*, belangrijk was voor expressie en een bindingsplaats van OsDof25. Dit werd bevestigd middels *in vitro* EMSA experimenten en in gist één-hybride analyse. Na GFP-tagging kon worden aangetoond dat OsDof25 een nucleair gelokaliseerd eiwit is in overeenstemming met zijn functie als nucleaire TF. Overexpressie en down-regulatie via RNAi van OsDof25 bevestigde haar rol bij de activering van de *OsC4PPDK* promotor. Dit suggereert dat OsDof25 een belangrijke rol speelt in het koolstof metabolisme, door te fungeren als transcriptionele regulator van de C4-routegenen.

In **hoofdstuk 4** werd een mogelijke rol van OsDof24 en OsDof25 bij de regulatie van de expressie van het GluB-1-gen uit rijst onderzocht met behulp van een transiënt expressiesysteem. Door co-transfectie van OsDof24 en OsDof25 overexpressieconstructen in combinatie met respectievelijk of een 245 bp GluB-1-promotor- of een GluB-1-promotorconstruct waaruit het gebied van -144 tot -119 was gedeleteerd, werden aanwijzingen verkregen dat OsDof24 en OsDof25 de GluB-1-promotor activeren door te binden aan twee potentiele Dof-bindingsmotieven binnen het 25 bp gebied van -144 tot -119 in

de GluB-1-promotor. Bovendien bevestigden in vitro EMSA (Elecrophoretic Mobility Shift Testen) experimenten dat het OsDof25 eiwit specifiek kon binden aan dit Dof bindingsmotief in de GluB-1-promotor. Tenslotte werd gevonden dat het stikstofgehalte van korrels van de OsDof24- of OsDof25-overexpressie en RNAi-transgene lijnen verschilde van dat van de controles. De resultaten in dit hoofdstuk indiceren dat rijst OsDof24 en OsDof25 een rol spelen in de regulatie van de GluB-1-promoter en dat zij de stikstofopslag beinvloeden via zaadopslageiwitten.

Samenvattend, het werk in dit proefschrift heeft geleid tot de karakterisering en een beter begrip van de functies van twee Dof-transcriptiefactoren die een rol spelen in het bepalen van de bloeitijd, de regulatie van het fotosynthesegen C4 PPDK en de expressie van zaadopslageiwitten in rijst.